



Dissertation zur Erlangung des Doktorgrades  
der Fakultät für Chemie und Pharmazie  
der Ludwig-Maximilians-Universität München

**The stress factor FKBP51 orchestrates pathways  
targeted by psycho-active drugs**

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aus

Düsseldorf, Deutschland

2013

### Erklärung

Diese Dissertation wurde im Sinne von § 7 der Promotionsordnung vom 28. November 2011 von Herrn Prof. F. Holsboer betreut und von Herrn Prof. H. Zorbas von der Fakultät für Chemie und Pharmazie vertreten.

### Eidesstattliche Versicherung

Diese Dissertation wurde eigenständig und ohne unerlaubte Hilfe erarbeitet.

München, 11.01.2013

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(Nils Gassen)

Dissertation eingereicht am 11.01.2013

1. Gutachter: Prof. Haralabos Zorbas
2. Gutachter: Prof. Florian Holsboer

Mündliche Prüfung am 03.06.2013

The work presented in this cumulative thesis was conducted in the research group of Dr. Theo Rein at the Max-Planck Institute of Psychiatry in Munich and comprises the results of four scientific publications:

**Gassen NC**, Hartmann J, Zschocke J, Stepan J, Kirmeier T, Wagner KV, Hafner K, Holsboer F, Ising M, Eder M, Schmidt MV, Rein T; FKBP51 shapes antidepressant action. (submitted).

**Gassen NC**, Hartmann J, Zschocke J, Maccarrone G, Hafner K, Kloiber S, Turck C, Lucae S, Holsboer F, Ising M, Schmidt MV, Rein T; FKBP51 inhibits GSK3 $\beta$  and amplifies the effects of distinct psychoactive drugs. (submitted).

Touma C, **Gassen NC**, Herrmann L, Cheung-Flynn J, Büll DR, Ionescu IA, Heinzmann JM, Knapman A, Siebertz A, Depping AM, Hartmann J, Hausch F, Schmidt MV, Holsboer F, Ising M, Cox MB, Schmidt U, Rein T (2011) FKBP5 shapes stress responsiveness: Modulation of neuroendocrine reactivity and coping behavior. *Biological Psychiatry*.

Schülke JP, Wochnik GM, Lang-Rollin I, **Gassen NC**, Knapp RT, Berning B, Yassouridis A, Rein T. (2010) Differential impact of tetratricopeptide repeat proteins on the steroid hormone receptors. *PLoS One*.



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## Abbreviations

4E-BP1	4E-binding protein 1
AD	antidepressant
Akt	(also PKB) protein kinase B
AMI	amitriptyline
Atg	autophagy related gene
CBD	calmodulin binding domain
c-Myc	myelocytomatosis oncogene
DEX	dexamethasone
FKBP51	FK506 binding protein 51 kDa
FKBP52	FK506 binding protein 52 kDa
FK1/2	FKBP12 like domain 1/2
FLX	fluoxetine
FoxO	forkhead box transcription factor
FST	forced swim test
GR	glucocorticoid receptor
GRE	glucocorticoid-response element
GSK3 $\beta$ / $\alpha$	glycogen synthase kinase 3 beta/ alpha
HIP	hippocampus
Hsc70	heatshock constitutive 70 kDa
Hsp90	heatshock protein 90 kDa
I $\kappa$ $\kappa$	inhibitor of $\kappa$ B kinase
LC3	light chain of microtubule associated protein
LEF	lymphoid enhancer-binding factor
LiCl	lithium chloride
MEF	mouse embryonic fibroblast
mTOR	mammalian target of rapamycin
NFAT	nuclear factor of activated T-cells
NF $\kappa$ B	nuclear factor Kappa-light-chain-enhancer of activated B-cells
p70 S6K	ribosomal protein S6 kinase, 70 kDa
PAR	paroxetine
PBMC	peripheral blood mononuclear cell
PFC	prefrontal cortex

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PHLPP	PH domain and leucine rich protein phosphatase
PP1	protein phosphatase 1
PP2A	protein phosphatase 2a
PP2B	protein phosphatase 2b, calcineurin
PPIase	peptidylprolyl isomerase
SNP	single nucleotide polymorphism
TCF	transcription factor
TPR	tetratricopeptide repeat
VPA	valproate
Vps34	class III phosphoinositide 3-kinase
Wnt	wingless-type MMTV oncogene integration-site

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## 1 Abstract

This thesis describes the impact of FK506 binding protein 51 (FKBP51) on molecular, cellular and physiological processes in neural systems and their resulting effects in behavioral phenotypes. FKBP51 is an important mediator of stress effects on various cellular and physiological processes. Initially this thesis contributed functional analyses of the FKBP51-dependent activity on the glucocorticoid receptor to studies that elucidated FKBP51's role in physiological stress response in mice and to studies that compared FKBP51 and related proteins with respect to their effect on steroid receptors.

In the first main part of this thesis, the influence of FKBP51 on Akt signaling and downstream events including autophagy was analyzed. Akt (protein kinase B) is a central hub of various signaling cascades. FKBP51 has been found to interact with Akt and to inhibit its catalytical activity by recruiting PHLPP phosphatase, which catalyzes the dephosphorylation of Akt at serine 473. As shown by *in vitro* and *in vivo* approaches, Akt affected downstream processes via mTOR (mammalian target of rapamycin) on autophagic markers in an FKBP51-dependent manner. These effects converged with those found for the antidepressants (ADs) amitriptyline, fluoxetine and paroxetine. In FKBP51-deficient mice treated with paroxetine, the forced swim paradigm showed no behavioral alterations indicative for efficacious AD actions, whereas wild-type littermates expressed an AD-like phenotype. Analysis of protein expression in the hippocampus and prefrontal cortex of these mice revealed an FKBP51-dependent regulation of Akt downstream effectors and autophagy markers in response to paroxetine exposure. Since autophagy has been recently linked to synaptic processes, it was assessed whether FKBP51 influences neurotransmission in combination with ADs. It was found that paroxetine exerted stimulatory effects on CA3-CA1 synapses of the hippocampus in mice expressing FKBP51, but not in FKBP51-deficient mice. To assess whether the molecular findings could be transferred to clinical settings, certain protein markers of peripheral blood mononuclear cells (PBMCs) from healthy subjects were analyzed. Similar correlations of FKBP51 levels with Akt serine 473 phosphorylation and autophagic markers Beclin1 and Atg12 (autophagy related gene 12) were found and an increased inducibility of these markers when these cells were cultivated *ex vivo* and exposed to ADs.

In the second part of the thesis, the focus was on the protein kinase glycogen synthase kinase 3  $\beta$  (GSK3 $\beta$ ) since this kinase modulates crucial neuronal functions. Accumulating evidence indicated an abnormal regulation of GSK3 $\beta$  in mood disorders. Here, a physical interaction of FKBP51 and GSK3 $\beta$  was found for the first time. This interaction resulted in elevated levels of serine 9 phosphorylation at GSK3 $\beta$ , which is the inactive form of GSK3 $\beta$ . Furthermore,

paroxetine and the mood stabilizer lithium shared common pathways with FKBP51 via inhibition of GSK3 $\beta$ . These findings were consolidated *in vitro* and *in vivo*. In a mass spectrometry based kinase assay, it was confirmed that lithium potently and directly inhibited the enzymatic activity of GSK3 $\beta$ . In addition, it also was found to directly inhibit GSK3 $\beta$ . Lithium and paroxetine strengthened the physical binding between FKBP51 and GSK3 $\beta$  as determined by co-immunoprecipitation (CoIP) experiments. Reporter gene assays measuring the activity of GSK3 $\beta$  by means of monitoring the activity of the downstream transcription factors TCF/LEF revealed a GSK3 $\beta$  inhibitory effect of ectopically expressed FKBP51 and of exposure to lithium or paroxetine. Moreover a synergistic effect of ADs and overexpressed FKBP51 was detected. In the absence of either GSK3 $\beta$  or FKBP51, reporter gene assays showed no inhibitory effects by lithium or paroxetine; in the absence of FKBP51, no elevation of inhibitory phosphorylation at pGSK3 $\beta$  (S9) was observed. Behavioral tests carried out in collaboration to determine AD effects in transgenic mice lacking FKBP51 showed no AD-like properties as compared to wild-type mice treated with either lithium or paroxetine. In human PBMCs, strong correlations between pGSK3 $\beta$  (S9) and FKBP51 protein expression were found. The stimulatory effects of lithium and paroxetine on pGSK3 $\beta$  phosphorylation at S9 were dependent on the FKBP51 expression level. To corroborate our molecular findings with clinical data, we used PBMCs from patients suffering from depression. In these cells, pGSK3 $\beta$  (S9) levels as well as its downstream substrate pTau (S622) were associated with clinical AD response.

In summary, this work provides new insights into the FKBP51 regulatory networks. FKBP51 crucially impacts various cell signaling events thereby affecting physiological and pathophysiological processes. This work corroborates FKBP51 in its pivotal role as a determinant of treatment response to psycho-active drugs.

## 2 Introduction

### 2.1 FKBP51 in psychiatric disorders and neurological diseases

Originally, Hsp90 (heat-shock protein 90) co-chaperone FKBP51 (FK506 binding protein 51) was identified as potentially relevant to stress-related diseases through its potent inhibition of glucocorticoid receptor (GR) function (Wochnik et al. 2005). Subsequently, FKBP51 was established as a crucial regulator of stress physiology by controlling the stress hormone axis. In genome-wide association studies, *fkbp5* (the gene encoding FKBP51) was linked to psychiatric disorders like major depression and post traumatic stress disorders (Binder et al. 2004).

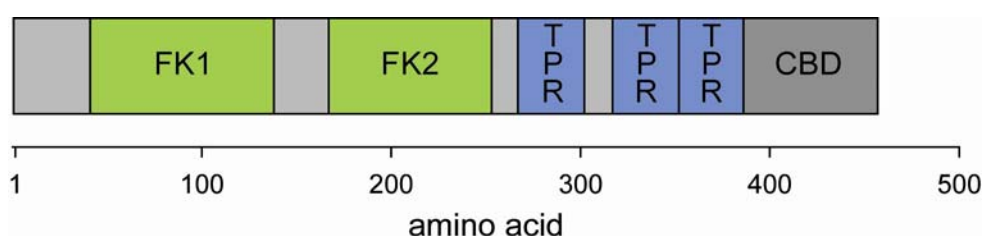
The closest homologue of FKBP51 is FKBP52 which features the same domain structure, but has divergent functional roles. For example, both proteins are regulators of steroid hormone receptor signaling, hormone binding, translocation as well as receptor maturation, but FKBP51 counteracts the stimulatory actions of FKBP52 and vice versa. During the last two decades, FKBP51 and FKBP52 emerged as key molecules in a variety of diseases including certain types of cancer, neurodegeneration, hormone-dependent diseases and stress-related illnesses (Wochnik et al. 2005; Storer et al. 2011). Binder et al. identified single nucleotide polymorphisms (SNPs) within *fkbp5* that significantly associated with antidepressant (AD) response and the recurrence of depressive episodes. These genotypes could be linked to elevated protein levels of intracellular FKBP51 with adaptive effects on GR function: patients carrying these SNPs had a decreased stress axis activity during depressive episodes (Binder et al. 2004). Recently Klengel et al. found that SNPs in the *fkbp5* gene play a decisive role in individuals developing posttraumatic stress disorders (PTSDs) when exposed to traumatic events in early childhood. These functional polymorphisms determines how long-range chromatin interactions between *fkbp5*'s transcription start site and intronic glucocorticoid-response elements (GREs) are changed by childhood-trauma through alterations of DNA-methylation; this underlies decreased or increased risk for stress-related psychiatric illnesses in adults. Through this mechanism, this DNA modification resulted in an enhanced transcription of stress-related genes leading to long-term imbalance of the stress hormone axis (Klengel et al. 2013). In addition to its importance in stress physiology, FKBP51 plays also an important role in neurodegenerative diseases like Alzheimer's: in addition to an abnormal aggregation of the microtubule-associated protein tau, age-related differences in the cell's chaperone repertoire were also found. In this context FKBP51, prevents tau clearance and modulates its phosphorylation status. *In vitro* FKBP51 isomerizes tau, and stabilizes microtubules (Jinwal et al. 2010). FKBP51 as an immunophilin plays a crucial part for

immune-related diseases and inflammation. It was reported that in a certain cell type of bone marrow of patients with rheumatoid arthritis *fkbp5* mRNA levels were elevated (Matsushita et al. 2010). Other studies provided evidence that FKBP51 modulates NF $\kappa$ B (nuclear factor Kappa-light-chain-enhancer of activated B-cells) signaling, resulting in changed expression of genes driven by NF $\kappa$ B transcription factors. This sheds light on FKBP51's link to immune regulation, inflammation, cell proliferation, metabolism, and hematopoiesis (Jiang et al. 2008; Baker et al. 2011). FKBP51 also influences NFAT (nuclear factor of activated T-cells) signal transduction via binding and inhibition of the NFAT regulating phosphatase calcineurin. This is thought to link FKBP51 to autoimmune diseases such as multiple sclerosis (Baughman et al. 1995).

Hence, FKBP51 is a versatile player in a multitude of cellular networks affecting physiological and pathophysiological processes. This is explained by the observations that FKBP51 as a co-chaperone interacts directly and indirectly with numerous proteins influencing downstream events (Storer et al. 2011).

## 2.2 FKBP51 – interaction, structure, signaling

FKBP51 was first described as binding partner of the progesterone receptor. Numerous studies demonstrate that beyond binding to steroid hormone receptors FKBP51 interacts with phosphatases and kinases resulting in changes of protein phosphorylation and modification of signaling molecules. As an Hsp90 co-chaperone, FKBP51 interacts with several proteins via Hsp90. Direct interaction of FKBP51 was demonstrated with Akt as well as with the protein phosphatase calcineurin. The interaction of these proteins with FKBP51 results in a change of their activity, with a series of consequences for downstream cascades (Pei et al. 2009; Li et al. 2002).



**Figure 1: Functional domains of human FKBP51**

FK1: domain with PPlase activity; FK2; homologues to FK1, but without enzymatic activity; TPR: domain responsible for binding to Hsp90; CBD: binding to calmodulin.

As depicted in figure 1 the FKBP51 protein consists of three main structural domains. The N-terminal FKBP12 like domain 1 (FK1) comprises the protein's enzymatic activity. As a peptidylprolyl isomerase (PPIase) FKBP51 catalyzes the *cis trans* conversion of peptidylprolyl bonds in proteins. The physiological relevance of this catalytic ability remained largely enigmatic. However, it proofed to be of some importance for the regulation of the stabilization of microtubules and phosphorylation of tau. Protein interactions described so far were not dependent on FKBP51's isomerization activity (Jinwal et al. 2010). The structural homologues FK2 domain does not contain an enzymatic function. As mentioned above, FKBP51 includes three tetratricopeptide repeat motifs that mediate Hsp90 bridged binding to client proteins. In proximity of the C-terminus of the FKBP51 molecule, a small region called calmodulin binding domain (CBD) enables binding of the calcium binding messenger protein calmodulin to FKBP51 (Sinars et al. 2003).

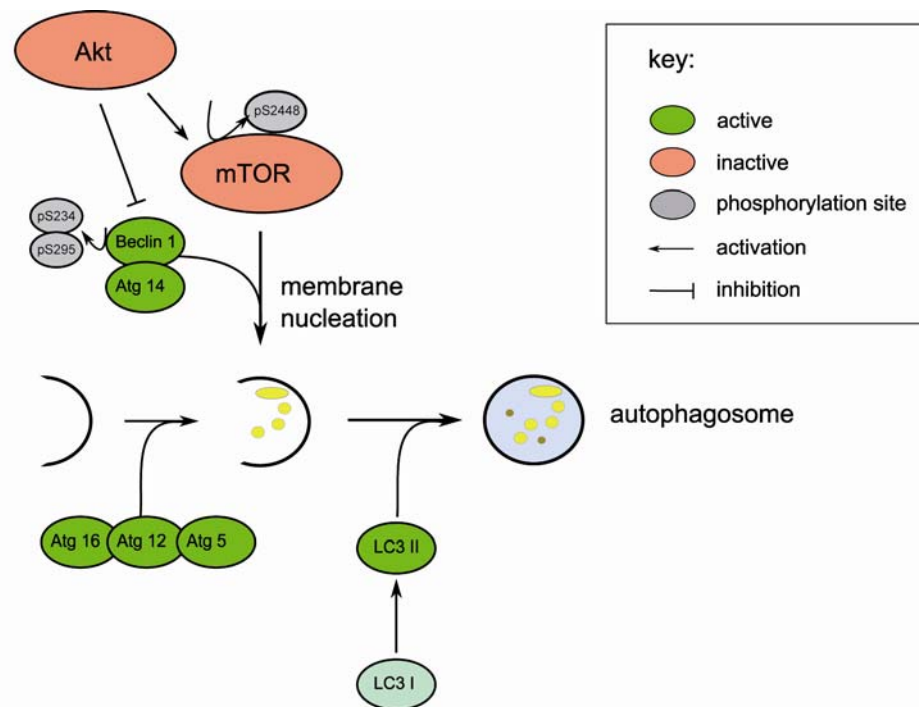
## 2.3 Pathways of FKBP51 and therapeutics

### 2.3.1 *Akt-mTOR pathway*

Pei et al. recently discovered that FKBP51 binds to the protein kinase Akt. Acting as a scaffolding protein, FKBP51 recruits protein phosphatase PHLPP (PH domain and leucine rich protein phosphatase) to Akt. PHLPP dephosphorylates Akt at serine 473 that leads to a reduced kinase activity and decreased phosphorylation of substrates including FoxO transcription factors or I $\kappa$ B (inhibitor of  $\kappa$ B kinase). Pei et al. described that FKBP51 protein expression levels determine the cancer patient's response to chemotherapeutics. Through modulation of Akt signaling, FKBP51 regulates pathways important for cell proliferation. Cells lacking FKBP51 proteins did not respond to chemotherapeutics targeting these pathways (Pei et al. 2009). Akt has multiple functions in a variety of cellular processes like cell survival, proliferation, transcription and glucose metabolism. In the context of psychiatric disorders, Akt seems to be a decisive factor for treatment response to a multitude of psychoactive drugs (Beaulieu et al. 2009). Psychotherapeutics were described as both inhibitors and stimulators of the Akt kinase, indicated by changes in the phosphorylation status at S473 or T308 that are both important for a fully active protein (Freyberg et al. 2010). Akt and the protein kinase mTOR were found to crosstalk. Akt phosphorylates mTOR at S2448 modulating its kinase activity (see also fig. 2). mTOR is a central player of cell growth and survival, cellular plasticity, and processing of damaged protein aggregates by autophagy. Treatment of the immunosuppressant drug rapamycin leads to inactivation of mTOR and



hence abolished kinase activity. Rapamycin was shown to increase autophagy and decrease apoptosis. Behavioral trials with rapamycin revealed antidepressant-like effects in a forced swim paradigm implying that mTOR is a potential therapeutic target for antidepressant drugs (Cleary et al. 2008).



**Figure 2: Regulating macroautophagy via Akt and mTOR**

Autophagy is triggered by inhibition of mTOR and Akt which in turn results in less inhibitory phosphorylation of Beclin1. Active Beclin1 in series with Atgs triggers membrane nucleation. To facilitate sequestration of autophagosomes LC3I is lipidated to LC3II.

### 2.3.2 Autophagy

Macroautophagy (here referred as to autophagy) is a conserved lysosomal process that regulates cytoplasmic integrity by elimination of damaged cell organelles and protein aggregates. Autophagy underlies the control of a multitude of regulators and signaling cascades initiating the engulfment of degradable molecules into double-membrane vesicles fusing to lysosomes. A series of Atgs (autophagy related genes), partly controlled by the mTOR kinase, regulate initial steps of autophagy processing. Recently, Wang et al. identified Atg6 (also beclin1) as a substrate of the Akt kinase. Beclin1 phosphorylation through Akt results in a decrease in autophagic flux and can proceed independently of mTOR signaling (Wang et al. 2012). For the assessment of autophagy, several markers can be used: lipidation of LC3-I to LC3-II (light chain of microtubule associated protein), expression levels of Vps34

(class III phosphoinositide 3-kinase), Beclin1, and certain Atgs (see Fig.2) (Funderburk et al. 2010). Autophagy was shown to play important roles in many cellular and physiological processes, reflecting its effects on energy balance and disposal of damaged macromolecules. Hernandez et al. linked autophagy to the regulation of neurotransmission. They found autophagic vacuoles in prejunctional dopaminergic axons associated with a reduced axonal volume, synaptic vesicle numbers as well as a decrease in evoked dopamine release (Hernandez et al. 2012).

Several studies indicate that autophagy can be targeted by different types of therapeutics. In 2011 Zschocke et al. showed that ADs amitriptyline (AMI) and citalopram elevate autophagic markers and finally lead to a higher degradation of macromolecules (Zschocke et al. 2011; Zschocke & Rein 2011).

Rubinsztein and colleagues put forward that the modulation of autophagy is a promising therapeutic target for the treatment of various diseases. The authors claimed that an increase in autophagic flux and the improved homeostasis can be advantageous for treatment of diverse illnesses such as distinct types of cancer and infectious and neurodegenerative diseases (Rubinsztein et al. 2012).

### ***2.3.3 GSK3 signaling***

Glycogen synthase kinase 3 (GSK3) is a serine and threonine kinase and is encoded by two genes, GSK3 $\alpha$  and GSK3 $\beta$ , with homologous structure but diverse function. GSK3 is established as a key regulator of a variety of signaling cascades and physiological processes and plays important roles in insulin and Wnt (wingless-type MMTV oncogene integration-site) signaling as well as in neurodegeneration. Unlike many other kinases involved in regulatory signals, GSK3 is active by default. GSK3 changes its active state only in the presence of external signals. GSK3's crystal structure revealed a catalytically active conformation in the absence of activation-segment phosphorylation (Dajani et al. 2001; Aoki et al. 2000). Aberle et al. described that through Wnt-signaling, GSK3 $\beta$  phosphorylates  $\beta$ -catenin, promoting its ubiquitination and successive degradation. Degradation only occurs when GSK3 $\beta$  is complexed with APC (adenomatous-polyposis-coli protein), Axin and  $\beta$ -catenin. When Wnt is active, the  $\beta$ -catenin destruction complex is inhibited via dishevelled allowing upregulation of  $\beta$ -catenin levels and subsequent elevation of the levels of transcription factors TCF (transcription factor) and LEF (lymphoid enhancer-binding factor) (Kimelman & Xu 2006). Among others, TCF and LEF target proliferative genes like c-Myc (myelocytomatosis oncogene) (Brüschle et al. 1993; Aberle et al. 1997).

### ***2.3.4 GSK3 and therapeutics - Two ways to inhibit GSK3 $\beta$***

The actions of lithium are manifold and have important cellular impacts as well as therapeutic effect for the treatment of bipolar disorders. Several molecular mechanisms underlying the effects of lithium have been identified so far. The therapeutic effects of lithium become manifest after a few weeks of treatment; plasma concentrations of about 1 mM are to be tightly controlled. One of the main targets of lithium is GSK3 $\beta$ . By inhibiting GSK3 $\beta$ , lithium modulates a multitude of cellular processes (Grimes & Jope 2001). To date, two mechanisms have been identified which explain the inhibitory effect of lithium on GSK3 $\beta$ : first, lithium inhibits GSK3 $\beta$  by direct interaction. Second, indirect inhibition of GSK3 $\beta$  through phosphorylation of S9 is triggered by lithium treatment (Jope 2003).

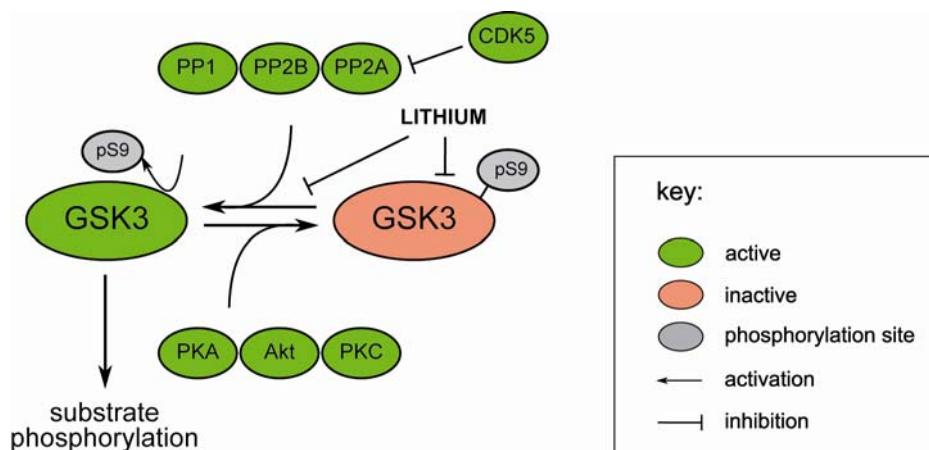
Direct effects of small-molecule inhibitors on certain enzymes usually involve binding to a cofactor or an important domain to block the catalytic activity. The direct effect of lithium on GSK3 $\beta$  is typical for this category of drugs. As a direct reversible inhibitor, lithium competes with Mg<sup>2+</sup> with an IC<sub>50</sub> of ~2 mM (Ryves & Harwood 2001). However the exact site lithium binds to remains elusive.

Indirect inhibition through post-translational modifications is a further mechanism to inhibit GSK3 $\beta$ 's activity by lithium. De Sarno and colleagues demonstrated that GSK3 $\beta$  as well as its close homologue GSK3 $\alpha$  is phosphorylated at a serine residue in their N-terminal region (S9, S21 respectively). An elevation of the phosphorylation of GSK3 results in a decreased enzymatic activity. Chronic treatment with lithium in rodents showed a marked increase in S9 phosphorylation at GSK3 $\beta$  (De Sarno et al. 2002). Kinases targeting this particular serine seem to vary depending on the cell type, condition, and treatment. This modification was shown to be catalyzed by Akt, protein kinase A (PKA) and protein kinase C (PKC). Reactivation of GSK3 $\beta$  and maintenance of a balanced phosphorylation status of GSK3 $\beta$  is regulated by phosphatases calcineurin (PP2B) and protein phosphatase 1 (PP1), dephosphorylate GSK3 $\beta$  at S9 (Jope 2003; Kim et al. 2009).

A recent publication by Omata et al. showed that in behavioral studies with rodents, inhibition of GSK3 $\beta$  has antidepressant-like effects. The authors used shRNA mediated knockdown of GSK3 $\beta$  in distinct brain regions. In diverse behavioral paradigms, they observed behavior comparable to mice treated with antidepressants (Omata et al. 2011).

Vice versa, ADs as well as other psychoactive drugs were found to modulate GSK3 $\beta$ 's activity by increasing phosphorylation of the inhibitory serine residue. For example, mood stabilizers of different classes like VPA and lamotrigine produced elevated levels of

pGSK3 $\beta$  (S9) (De Sarno et al. 2002; Tomaszewicz et al. 2006). The ADs imipramine and fluoxetine (FLX), also increased pGSK3 $\beta$  (S9) levels in mouse brains, when administered in combination with risperidone (Li et al. 2007).



**Figure 3: Pathways involved in GSK3 $\beta$  inhibition**

In active state, GSK3 $\beta$  catalyzes the phosphorylation of many substrate proteins. GSK3 $\beta$  is inhibited by phosphorylation at serine in the N-terminal domain. This modification can be catalyzed by diverse kinases: Akt, PKA: protein kinase A, PKC: protein kinase C. The inactive phospho-serine can be reactivated by protein phosphatases: PP2B: calcineurin, PP2A: protein phosphatase 2A, PP1: protein phosphatase 1. Cyclin dependent kinase 5 can elevate inhibitory phosphorylation (S9) by inhibiting PP2A or PP1. Lithium increased phosphorylation at S9 or inhibits GSK3 $\beta$  directly.

## 2.4 Aims of the study

The goal of this thesis was to shed light on FKBP51 modulated molecular pathways and convergent signaling events driven by psychoactive drugs.

Several studies point to a critical role of FKBP51 in Akt signaling and hence on autophagic pathways (Pei et al. 2009; Eskelinen 2011). However, molecular modes of action of FKBP51 still remain enigmatic. Foremost, the identification of novel interaction partners of FKBP51 and the assessments of the cellular and physiological impacts of these complexes are of greatest importance. In order to understand the role of FKBP51 in the context of AD treatment and response, potentially converging pathways of FKBP51 and ADs were analyzed *in vitro* and *in vivo*: experiments that aimed at addressing these questions included, the investigation of marker molecules indicative of autophagy, the forced swim test (FST) and electrophysiological monitoring using FKBP51-deficient animals.

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As described in the literature, FKBP51 was found to interact with and inhibit Akt kinase by recruiting the phosphatase PHLPP. Putative effects of FKBP51 on one of Akt's most prominent substrates, GSK3 $\beta$  were not evaluated so far (Pei et al. 2009). Therefore, the physical interaction of FKBP51 to GSK3 $\beta$  was also characterized in detail. Next, the underlying mechanisms and functional relevance including the role of CDK5 were evaluated. Various studies evidenced the effects of ADs and mood stabilizers, especially lithium, on GSK3 $\beta$  activity. Therefore, the impact of FKBP51 on GSK3 $\beta$  in combination with psychoactive drugs was analyzed *in vitro* and *in vivo*.

Finally, to translate the findings to the clinical situation, peripheral blood mononuclear cells (PBMCs) were used to correlate FKBP51 expression levels to the expression of the distinct marker molecules.

### 3 Results

The following section summarizes the results obtained from the manuscripts, highlighting the contributions of this thesis. Full length results including supplementary information and the applied methods are presented in the articles (see appendix).

The first part of the thesis contributed to the characterization of FKBP51 in cellular and physiological stress response. In the manuscript of Schülke et al. 2010 *Differential impact of tetraatricopeptide repeat proteins on the steroid hormone receptors* the effect of seven Hsp90 binding proteins on the function of the five steroid receptors was determined. The contribution of this thesis was to explore the impact on GR response to stress hormones by GRE driven reporter gene assays in FKBP52-deficient mouse embryonic fibroblasts (MEFs). Loss of FKBP52 led to a decreased GR activity (see Fig. 12, Schülke et al. 2010). The manuscript by Touma et al. 2011 *FK506 binding protein 5 shapes stress responsiveness: modulation of neuroendocrine reactivity and coping behavior* characterized FKBP51-deficient mice in different behavioral paradigms. It revealed that under basal conditions, FKBP51 knock outs showed no significant differences to wild-type animals. When FKBP51 knock-out mice were exposed to different types of acute stress, an increase in active stress coping behavior was observed. Loss of FKBP51 diminished HPA-axis reactivity and resulted in changes of GR expression in response to stressors. The contribution of this thesis was to determine the effect of FKBP51 deficiency on GR function; reporter gene assays in FKBP51<sup>-/-</sup> and FKBP51<sup>+/+</sup> MEFs indicated increased GR hormone affinity in cells devoid of FKBP51. When FKBP51 was ectopically expressed in FKBP51<sup>+/+</sup> MEFs, hormone affinity was decreased (see Fig. 1 A&B; Touma et al. 2011). Ectopic expression of FKBP52 in MEF FKBP52<sup>-/-</sup> and FKBP51 into FKBP51<sup>-/-</sup> cells respectively were carried out to mimic wild-type protein status that excluded effects by compensatory mechanisms often found in knock out cells.

In the third manuscript described here, Gassen et al. *FKBP51 shapes antidepressant action*, (in the following referred as to MS-I), the FKBP51-dependency of antidepressant effects on Akt-pathways including autophagy was investigated in cells, in mice and in humans. First, the interacting domains of FKBP51 with Akt1 and PHLPP phosphatase were fine-mapped. HEK (human embryonic kidney) 293 cells were chosen for co-immunoprecipitation (CoIPs) experiments, because they expressed sufficient amounts of each protein to be analyzed and could be efficiently manipulated. FKBP51 interacted with Akt1 independent of Hsp90 and its

catalytic PPIase activity since FKBP51 TPRmut and PPImut constructs precipitated equally efficiently with Akt1 compared with full-length FKBP51. Interaction was mapped mainly to the FK1 domain of FKBP51. FKBP52 interacted with Akt1 but not with PHLPP. The functional consequence of the interaction of FKBP51 with Akt1 and PHLPP was studied by Western Blot analyses of pAkt S473 and T308, indicating active forms of Akt, and in addition of the Akt downstream target pFoxO3a (S318). A lack of binding to PHLPP phosphatase as shown for FKBP52 and FKBP51  $\Delta$ FK1  $\Delta$ FK2 also resulted in a loss of Akt dephosphorylation (S473) and downstream effects on pFoxO3a (S318) and its transcriptional activity. (see Fig.1 A-C; Gassen et al. MS-I). To evaluate the functional relevance of the complex formation between FKBP51 and Akt1, Western Blot analyses were performed to determine the levels of downstream proteins, mTOR substrates 4e-BP1 and p70-S6K and the autophagy markers Atg12, Beclin1 and LC3II/LC3I using FKBP51<sup>-/-</sup> and Akt1/2<sup>-/-</sup> MEFs. The results revealed a regulation of the marker proteins only in presence of FKBP51 and Akt1/2 (Fig.2; Gassen et al. MS-I). In addition, the impact of the ADs AMI, FLX and paroxetine (PAR) on these pathways as well as on autophagosome formation was assessed. Western Blot analyses with lysates from rat cortical astrocytes ectopically expressing FKBP51 or empty vector were carried out. Since ADs had stimulatory effects on Akt1 (S473) dephosphorylation and on autophagy markers, ectopic FKBP51 amplified the effects of ADs on these molecules (Fig.3 B, C, E-I; Gassen et al. MS-I). FKBP51 deficient mice were evaluated in the FST to characterize the role of FKBP51 in AD response *in vivo*. Animals were treated for 45 min with PAR (10 mg/kg) and underwent behavioral testing. Subsequently, proteins were extracted from hippocampi and prefrontal cortices for Western blot analysis. The FST showed an effect of PAR in wild-type mice, but no significant change in behavior in FKBP51<sup>-/-</sup> mice. Protein analysis revealed that PAR triggered the elevation of pAkt (S473) levels and autophagy markers only in FKBP51<sup>+/+</sup> but not FKBP51<sup>-/-</sup> mice (Fig.4; Gassen et al. MS-I). In collaboration, hippocampal slices of FKBP51<sup>+/+</sup> and FKBP51<sup>-/-</sup> mice were used to assess neurotransmission in the CA1-CA3 regions. Neuronal activity of the CA1 area was measured by means of voltage-sensitive dye imaging (VSDI). PAR treatment (10  $\mu$ M) of brain slices excited neurotransmission in CA1. This was only observed in brain slices of FKBP51<sup>+/+</sup> mice (Fig.5; Gassen et al. MS-I). In addition, levels of pAkt (S473) as well as autophagy markers were analyzed in PBMCs obtained from healthy male subjects. Protein expression levels of FKBP51 strongly correlated with the expression of the autophagy markers Beclin1, Atg12, and to a lesser extent with LC3B-II/LC3B-I; FKBP51 correlated negatively with pAkt (S473) (Fig.6; Gassen et al. MS-I). AD effects in human cells were measured by *ex vivo*

cultivation of PBMCs and treatment with ADs. Applied drug concentrations were based on standard therapeutic drug serum levels (Hiemke et al. 2011). The inducibility of autophagy markers by AMI, FLX and PAR correlated significantly with the FKBP51 expression level; Beclin1 and LC3B-II/LC3B-I and for Atg12 only when stimulated with PAR. Inducibility of pAkt (S473) by ADs negatively correlated with protein expression of FKBP51 (Fig.7; Gassen et al. MS-I).

The forth manuscript by Gassen et al. *FKBP51 inhibits GSK3 $\beta$  and amplifies the effects of distinct psychoactive drugs*, (in the following referred to as MS-II) focused on the effect of FKBP51 on GSK3 $\beta$  and how this impacts on downstream pathways and the action of psychoactive drugs in cells, mice and humans. Most of methods were performed in analogy to MS-I. A detailed mapping and functional interaction analysis of FKBP51 with GSK3 $\beta$  preceded a detailed characterization of cellular and physiological effects by a multitude of *in vitro* and *in vivo* experiments. Interaction of FKBP51 with GSK3 $\beta$  was mediated predominantly by FKBP51's FK1 domain, whereas FKBP51's catalytical activity as well as binding to Hsp90 had no influence. Functional assays revealed that only mutants of FKBP51 capable to bind GSK3 $\beta$  triggered increased pGSK3 $\beta$  (S9) levels. FKBP52 interacts with GSK3 $\beta$ , but induced no change in pGSK3 $\beta$  (S9) or TCF/LEF mediated transcription (Fig.1 A-C; MS-II). The effect of FKBP51 and FKBP52 on pGSK3 $\beta$  (S9) and downstream targets pTau (S396; S622) and  $\beta$ -catenin was analyzed in HEK cells after ectopic expression of the immunophilins. FKBP51 overexpression resulted in the upregulation of pGSK3 $\beta$  (S9) and proteins downstream modulated by GSK3 $\beta$  like pTau (S396; S622) and  $\beta$ -catenin. FKBP52 exerted no effect on the expression levels of the analyzed proteins apart from a significant decrease in pAkt (S473) when highly overexpressed (Fig.1; Gassen et al. MS-II). To analyze the effects of FKBP51/52, alone or in combination with psycho-active drugs, on GSK3 $\beta$  dependent transcription, TCF and LEF driven reporter gene assays were performed in HEK cells, rat primary cortical astrocytes, neurons, and in MEF cells lacking FKBP51 or GSK3 $\beta$ . FKBP51 overexpression produced stronger induction of reporter activity in combination to lithium, PAR or VPA than in the absence of drugs. The stimulatory effects of psycho-active drugs on TCF/LEF driven reporter genes were abolished upon deletion of FKBP51. As in HEK cells, FKBP52 also produced no effect on TCF/LEF signaling in cortical astrocytes (Fig.1 C, Fig.3 A,B; MS-II).

Since the inhibitory effect of FKBP51 on Akt cannot explain the increase in pGSK3 $\beta$ , a possible interaction of FKBP51 with CDK5, another GSK3 $\beta$  regulating kinase, was



investigated. Co-immunoprecipitation experiments were carried out using HEK cells ectopically expressing FLAG-tagged FKBP51 that allowed precipitation of FKBP51 via anti-FLAG conjugated magnetic beads. CDK5 was co-immunoprecipitated with FKBP51. The role of CDK5 in regulating GSK3 $\beta$  activity was determined by the use of FKBP51-deficient MEF cells and ectopic expression as well as specific pharmacological inhibition of CDK5 by roscovitine. Inhibition by roscovitine resulted in markedly decreased levels of pGSK3 $\beta$  (S9). Ectopic expression of CDK5 led to an increase of pGSK3 $\beta$  (S9) levels in wild-type MEFs, but not in MEFs devoid of FKBP51 (Fig.2; MS-II). Effects of psycho-active drugs were observed also at the level of protein-protein interaction. Lithium and to lesser extent PAR enhanced the interaction between GSK3 $\beta$  and FKBP51 (Fig.4 F; MS-II). Measurements of the catalytic activity of GSK3 $\beta$  in a mass spectrometry based kinase assay (performed according to Bowley et al. 2005) revealed a decline of GSK3 $\beta$ 's kinase activity when the kinase reaction was complemented with lithium or PAR (Fig.4 G, H; MS-II). Effects of psycho-active drugs on pGSK3 $\beta$  (S9) phosphorylation as well as on TCF/LEF dependent transcription in wild-type MEF cells and in the absence of FKBP51 or GSK3 $\beta$ , showed that FKBP51 is crucial to mediate the effects on phosphorylation of pGSK3 $\beta$  (S9) and downstream events and GSK3 $\beta$  played an essential role in TCF/LEF signaling. Interestingly, VPA induced pGSK3 $\beta$  (S9) levels as well as improved TCF/LEF signaling even in absence of FKBP51 (Fig. 3B, Fig.4 A-E; MS-II). *In vivo* experiments were performed in order to assess the response to lithium (85 mg/kg) of mice in dependence of the FKBP51 expression status at the behavioral and protein expression level (refer to MS-I). In the FST, lithium exerted an AD-like effect in FKBP51<sup>+/+</sup> animals, whereas FKBP51<sup>-/-</sup> mice displayed an impaired response to lithium. Western blot analyses performed with HIP, PFC and blood samples showed elevated pGSK3 $\beta$  (S9) levels after treatment with lithium or PAR only in FKBP51 wild-type animals (Fig.5; MS-II). Correlations of proteins expressed in PBMCs of healthy male subjects and *ex vivo* cultivation in addition to stimulation with psycho-active drugs were also performed. FKBP51 protein expression levels positively correlated with pGSK3 $\beta$  (S9) and  $\beta$ -catenin. The inducibility of pGSK3 $\beta$  (S9) after treatment with lithium or PAR, but not VPA, were correlated with FKBP51 expression (Fig.6 A-E; MS-II). PBMCs obtained from 56 patients with major depression, as a part of the MARS (Munich Antidepressant Response Signature) project, were analyzed for protein expression levels of pGSK3 $\beta$  (S9) and pTau (S622) by a commercial chip based protein-array. It showed that clinical response represented by the Hamilton score of depression (HMD) evaluated before and after AD therapy could be associated with increased pGSK3 $\beta$  (S9) and significantly with decreased pTau (S622) (Fig.7 A,B; MS-II).

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## 4 Discussion

FKBP51 emerged from genotype association studies as prominent factor in stress-related diseases such as depression and in the response to antidepressant treatment. It was included in candidate genotype studies originally because of its impact on GR (Wochnik 2005, Binder 2004). Subsequent studies, to which this thesis contributed, provided further insight into FKBP51's role in stress physiology and in the regulation of other signaling pathways (Binder et al. 2004; Jinwal et al. 2010; Klengel et al. 2013).

Binder et al. reported that FKBP51 genotypes associated with higher levels of FKBP51 proteins are also linked to improved clinical response to treatment with ADs (Binder et al. 2004). Accordingly, results provided in MS-I and MS-II show that higher levels of FKBP51 enhance the effect of psycho-active drugs such as ADs on Akt and GSK3 $\beta$  and downstream pathways. The protein kinases Akt and GSK3 $\beta$  have been reported as targets of ADs and mood stabilizers before (Beaulieu et al. 2009). Based on the findings of *in vitro* and *in vivo* experiments of MS-I and MS-II, the protein expression status of FKBP51 determines whether and how psycho-active substances alter Akt- and/or GSK3 $\beta$ -directed pathways. Thus, the FKBP51 expression level might determine cell-specific differences in drug effects on Akt phosphorylation described in literature (Beaulieu et al. 2009; Pei et al. 2009; Pei et al. 2010). Apart from FKBP51, Akt's catalytic activity is orchestrated by multiple factors, the combination of which might be decisive determinants for ADs exerting either inhibitory or stimulatory effects on Akt kinases.

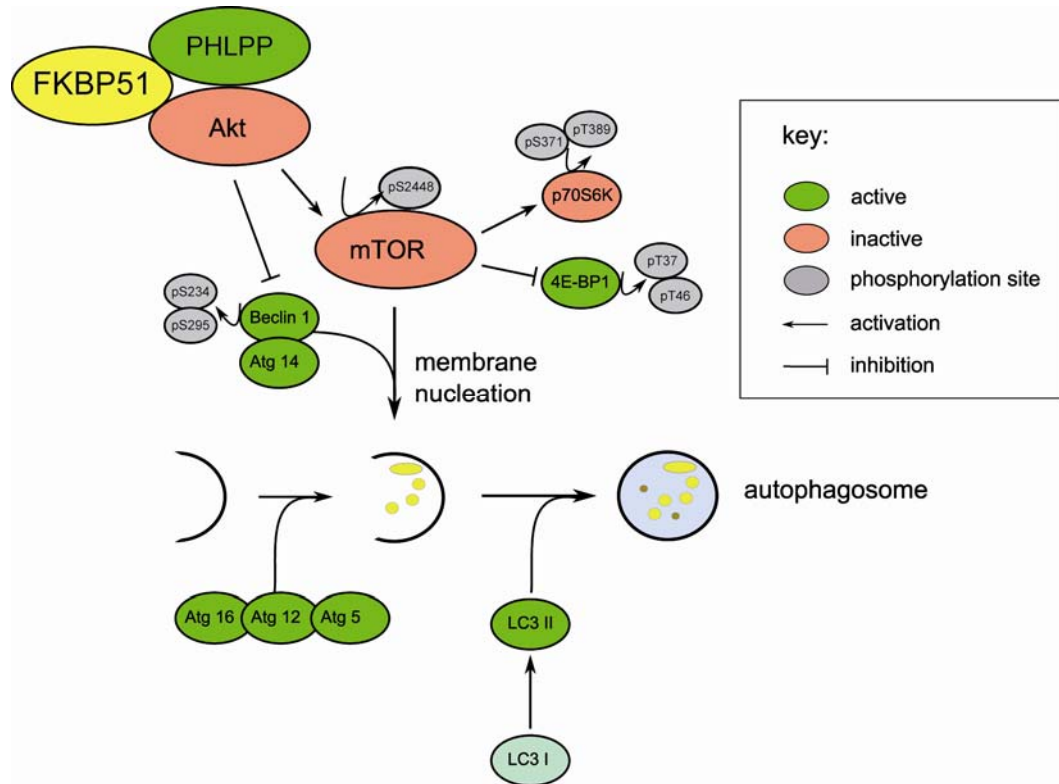
Considering the drug relevant mechanism of action of FKBP51, the protein-protein interaction analyses described in MS-I support the role of FKBP51 as scaffolding protein that recruits the phosphatase PHLPP to Akt thereby facilitating Akt1 dephosphorylation. The FK1 domain of FKBP51 interacted with both Akt and PHLPP. This interaction is independent of FKBP51's PPIase activity and of binding to Hsp90. Although Akt and PHLPP were shown to be chaperoned by Hsp90 (Sinars et al. 2003; Sato et al. 2000), an Hsp90 independent interaction with FKBP51 is not unexpected, as intrinsic chaperone activity has been described for FKBP51 (Pirkl & Buchner 2001). Interaction analyses also revealed FKBP52 interacting with Akt, but not with PHLPP. Thus, FKBP52 did not directly affect pAkt (S472) levels. FKBP51 and FKBP52, although closely homologous, have divergent functions for neurite outgrowth (Quintá et al. 2010), regulation of microtubules (Jinwal et al. 2010), and the control of corticosteroid receptors (Wochnik et al. 2005; see also Schülke et al. 2010 and Touma et al. 2011).

In MS-II, physical binding of FKBP51 and FKBP52 to GSK3 $\beta$  is presented for the first time. Even though both immunophilins bound to GSK3 $\beta$ , FKBP52 played a functionally antagonistic role to FKBP51. Binding of GSK3 $\beta$  to FKBP51 was shown to be also mediated largely via the FK1 domain of FKBP51; interestingly, binding was independent of Hsp90 binding of FKBP51, although GSK3 $\beta$  is chaperoned by Hsp90 with the result of priming phosphorylation at Y216, necessary for GSK3 $\beta$  full catalytical activity (Lochhead et al. 2006).

By interacting with various protein phosphatases and kinases FKBP51 is capable to orchestrate posttranslational modifications at proteins and transcription factors of entire signaling networks. As presented in MS-I and MS-II, FKBP51 regulates promoters driven by the transcription factors FoxO3a and TCF/LEF via modulation of Akt1 and GSK3 $\beta$  activity. Originally, we hypothesized that the inhibitory effect of FKBP51 on Akt kinase should result in decreased pGSK3 $\beta$  (S9) levels, since GSK3 $\beta$  is an established substrate of Akt (Pei et al. 2009). Phosphorylation of Akt at T308 is required for Akt-directed phosphorylation of pGSK3 $\beta$  (S9) (Pan et al. 2011). Unexpectedly, FKBP51 appeared to inhibit GSK3 $\beta$  by direct interaction resulting in elevated levels of pGSK3 $\beta$  (S9) in parallel to an inhibition of Akt1 as mirrored by reduced pAkt (S473) and no effect on phosphorylation at pAkt (T308). The inhibitory effect on GSK3 $\beta$  involves CDK5 as a regulator of GSK3 $\beta$ ; CDK5 is presented here as a novel interaction partner of FKBP51. GSK3 $\beta$  and CDK5 were found to play complementary roles in diverse cellular processes and disorders such as neurodegenerative diseases (Maldonado et al. 2011). In Alzheimer's disease, both kinases were identified as regulators of Tau phosphorylation and Tau clearance (Plattner et al. 2006). Jinwal et al. described that FKBP51 mediates phosphorylation of Tau, pointing to another mechanism that linked FKBP51 to CDK5 and GSK3 $\beta$  (Jinwal et al. 2010).

The modulation of Akt1 activity through FKBP51 has an important impact on autophagy (see Fig. 4). Several studies linked autophagy to the pathology of diverse diseases. Furthermore, autophagy can be triggered by diverse therapeutics such as ADs (Zschocke & Rein 2011; Zschocke et al. 2011). FKBP51 might link stress physiology to autophagic processes. Laane et al. reported that treatment with the synthetic glucocorticoid dexamethasone induced the autophagic markers Beclin1 and Atg12 *in vitro* (Laane et al. 2009). It is well known that the manipulation of autophagy has far reaching consequences that ultimately precipitate in cellular and physiological events. Transgenic mice lacking Atg7, a crucial regulator in autophagosome formation, had a significant loss of body weight compared to wild-type animals (Zhang et al. 2009). This is in line with the observations that FKBP51<sup>-/-</sup> mice weighed

less compared to wild-type littermates (Hartmann et al. 2012). Thus, FKBP51 induced autophagy might at least partially account for the changes in mouse body weight in FKBP51<sup>-/-</sup> mice.



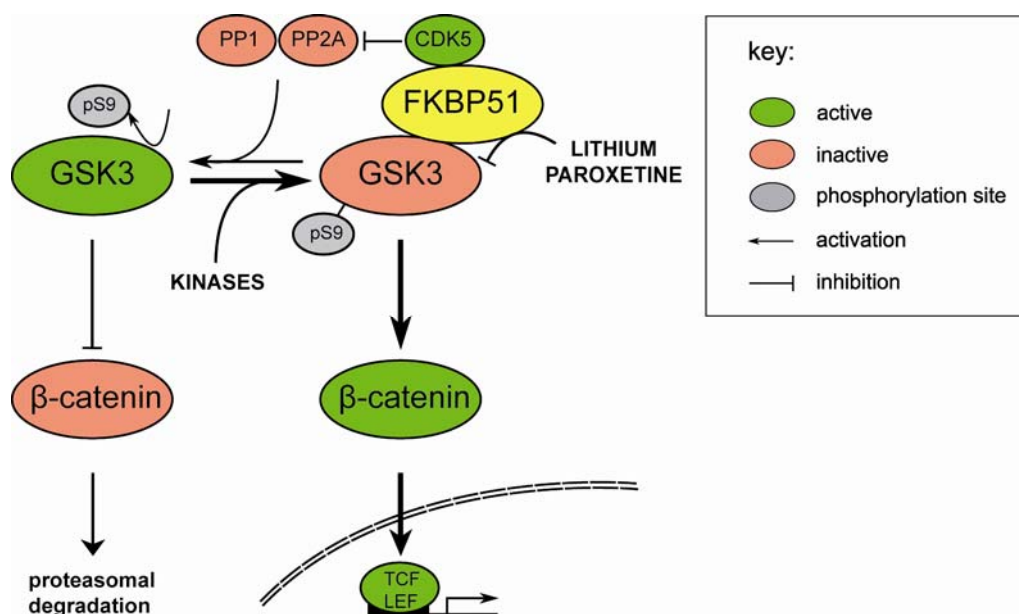
**Figure 4: FKBP51 regulates macroautophagy**

FKBP51 recruits Akt to PHLPP, which in turn inhibits Akt by dephosphorylation. Less efficient Akt kinase activity results in a decrease of activated mTOR; thereby, the two Beclin-inhibitors Akt and mTOR are inhibited. Beclin1 triggers membrane nucleation in concert with a series of Atgs. Sequestration of autophagosomes is facilitated by lipidation of LC3I to LC3II

Akt and GSK3 $\beta$  activity is affected by mood stabilizers and ADs, although with different compound specific outcomes (Beaulieu et al. 2009). In MS-I and MS-II the effects of diverse psycho-active drugs on Akt and GSK3 $\beta$  phosphorylation and activity as well as on downstream targets were abolished in FKBP51-deficient mice and cell-lines. For instance, treatment with the AD PAR resulted in the regulation of Akt- and GSK3 $\beta$ -dependent pathways *in vitro* and *in vivo*, in dependence of FKBP51. For the first time it was shown that PAR directly inhibited GSK3 $\beta$ , suggesting that this enzyme is a potential mediator of PAR's therapeutic actions (see Fig. 5).

This thesis presents FKBP51 as a novel regulator of autophagy and GSK3 $\beta$ , and links FKBP51 expression to AD/ mood stabilizer response. Analysis of clinical samples confirmed that FKBP51 as well as GSK3 $\beta$  and its substrate Tau might serve as predictive markers for AD response.

The results of this thesis put forward that FKBP51 determines response to ADs and mood stabilizers by shaping cellular pathways like Akt1 and GSK3 $\beta$  signaling; inhibition of GSK3 $\beta$  or Akt improved AD effects. This thesis could show both proteins to be regulated by FKBP51, thereby promoting FKBP51 as a pivotal determinant in therapeutic effects through ADs. The here established impact of FKBP51 on protein kinases, signal transduction and autophagic processes significantly expands the range of actions of this versatile protein.



**Figure 5: Model of the multifarious ways to inhibit GSK3 $\beta$  pathways orchestrated by FKBP51**

FKBP51 governs inhibition of GSK3 $\beta$  by enabling CDK5 to block phosphatases PP2A and PP1. This results in decrease of dephosphorylation which shifts the equilibrium to more inhibitory phosphorylation of GSK3 $\beta$  at S9. Less  $\beta$ -catenin will be phosphorylated and therefore will not undergo proteasomal degradation.  $\beta$ -catenin binds TCF/LEF and activates promoters driven by these transcription factors. Inhibition of GSK3 $\beta$  by psychoactive drugs can be direct, but is significantly enhanced through strengthening the interaction of FKBP51 with GSK3 $\beta$  leading to increased phosphorylation at S9 and stronger inhibition.

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## 6 Appendix

This section contains the reprints of the following articles:

I

**Gassen NC**, Hartmann J, Zschocke J, Stepan J, Kirmeier T, Wagner KV, Hafner K, Holsboer F, Ising M, Eder M, Schmidt MV, Rein T; FKBP51 shapes antidepressant action. (submitted).

II

**Gassen NC**, Hartmann J, Zschocke J, Maccarrone G, Hafner K, Kloiber S, Turck C, Lucae S, Holsboer F, Ising M, Schmidt MV, Rein T; FKBP51 inhibits GSK3 $\beta$  and amplifies the effects of distinct psychoactive drugs. (submitted).

III

Touma C, **Gassen NC**, Herrmann L, Cheung-Flynn J, Büll DR, Ionescu IA, Heinzmann JM, Knapman A, Siebertz A, Depping AM, Hartmann J, Hausch F, Schmidt MV, Holsboer F, Ising M, Cox MB, Schmidt U, Rein T (2011) FKBP5 shapes stress responsiveness: Modulation of neuroendocrine reactivity and coping behavior. *Biological Psychiatry*.

IV

Schülke JP, Wochnik GM, Lang-Rollin I, **Gassen NC**, Knapp RT, Berning B, Yassouridis A, Rein T. (2010) Differential impact of tetratricopeptide repeat proteins on the steroid hormone receptors. *PLoS One*.

## Curriculum Vitae

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09/2002 – 09/2005 University of Applied Sciences, Bonn, degree: Bachelor of Science; 07/2005

### Awards

Mifek-Kirscher-Preis 2012; collaborative work: Kirmeier T, Knob M, **Gassen NC**; Title of the awarded project: FKBP51: A Novel Regulator for Multiple Sclerosis (MS) Drugs.

### List of publications

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**Gassen NC**, Hartmann J, Zschocke J, Maccarrone G, Hafner K, Kloiber S, Turck C, Lucae S, Holsboer F, Ising M, Schmidt MV, Rein T; FKBP51 inhibits GSK3 $\beta$  and amplifies the effects of distinct psychoactive drugs. (submitted).

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München

Nils C. Gassen

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## **Acknowledgements / Danksagung**

Als erstes danke ich Theo für das interessante Forschungsprojekt, die exzellente Betreuung meiner Arbeit und dafür immer Zeit für Fragen genommen zu haben, auch wenn das oftmals Stunden dauerte. Vielen Dank auch für die große Unterstützung und zahlreichen Diskussionen. Legendär sind da die Diskussionen mit Jan und Regina über die Regulation der Gaussia. Vielen Dank auch für die viele Hilfe!

Vielen Dank Herr Jürgen Jochen Günter Zschocke. Es war mir eine Ehre. Wärst du nur geblieben, wir hätten Großes schaffen können...haben wir auch so, aber vielleicht noch Größeres. Vielen Dank für deine große Hilfe!

Ein riesengroßes Dankeschön an Käthe, für deine viele Hilfe und für lustige Zellkulturstunden. Du bist der Meister der Blots.

Vielen Dank an meine Kollegen aus der AG Rein. Ihr seid super. Vielen Dank, besonders für eure Unterstützung in den letzten Tagen meiner Dissertation. Erst einmal vielen Dank Vanessa, für das gute Absichern, nicht nur als Kletterpartnerin auch als Mutter unserer Tochter. Vielen Dank Kirmi für zahlreiche Diskussionen und Ideen. Ich danke natürlich Anna und Käthe, ohne euch würden wir untergehen. Madeleine, du solltest bei uns bleiben, es macht Spaß mit dir an einem Projekt zu arbeiten. Vielen Dank auch an Anja und Svenja, wenn es nötig sein sollte, spendiere ich mal eine Flasche Sojasoße. Lorenz und Bozo – vielen Dank für die fast epischen Tischtennisschlachten. Danke Andreas für die Mass-Spec-Assays. Madita, Madita... .

Danke auch an die Ex-AG Reinler: Nicki, Tatjana, Jan, Regina, Kristina, Birgit, Yuchen ...

Vielen Dank an meine Kollegen aus anderen Gruppen für die gute Zusammenarbeit und zahlreiche Diskussionen: Jakob, Klaus, Mathias (Schmidt), Jens, Matthias (Eder), Joachim, Matthias (Knob), Yi-Chun, Elmira, Carsten, Marcus, Guiseppina, Andreas, Chris, Christian, Alex, Sebastian, Ulli, Bozo, Leonie, Dominik, Irina, Christine...

Vielen Dank Prof. Holsboer für die Möglichkeit, an diesem Institut meine Forschungsarbeit durchführen zu können, dadurch war es mir möglich zu erleben, wie es ist, ein Teil des Max-Planck Instituts für Psychiatrie zu sein. Vielen Dank auch an Prof. Zorbas für die Betreuung meiner Arbeit an der Fakultät für Chemie und Pharmazie. Danke auch an die Mitglieder des Prüfungskomitees.

Vielen Dank Alwine für deine große Unterstützung und dein Verständnis. Danke auch an Helene, dass du mir noch die Zeit gegeben hast meine Dr. Arbeit fertigzuschreiben.



## **FKBP51 inhibits GSK3 $\beta$ and amplifies the effects of distinct psychoactive drugs**

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## Abstract

Psychoactive drugs such as lithium and antidepressants target glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ). However, the functional integration with other factors relevant to drug action is poorly understood. We here show protein interaction between GSK3 $\beta$  and FK506 binding protein (FKBP) 51, which has been genetically linked to the actions of antidepressants. This interaction is accompanied by increased phosphorylation of GSK3 $\beta$  at serine 9 (pGSK3 $\beta$ ) and downstream effects on beta catenin, tau phosphorylation and transcriptional activity of TCF/LEF. Deletion of GSK3 $\beta$  abolished the effect of FKBP51 on TCF/LEF. Lithium enhanced the interaction between FKBP51 and GSK3 $\beta$  and reporter gene assays revealed a synergistic effect of lithium or the antidepressant paroxetine with FKBP51 on TCF/LEF. Kinase activity assays revealed also a direct inhibition of GSK3 $\beta$  by paroxetine. The increase in pGSK3 $\beta$  observed in cells and mice after exposure to paroxetine or lithium was blunted upon deletion of FKBP51; deletion of FKBP51 also attenuated the effect of lithium in the forced swim test. In *ex vivo* cultivated human peripheral blood mononuclear cells (PBMCs) from healthy volunteers, the increase of pGSK3 $\beta$  upon treatment with lithium or paroxetine correlated with FKBP51 expression. Clinical improvement of drug-treated depressive patients correlated with protein markers of GSK3 $\beta$  pathway activity in their PBMCs. These data link FKBP51 to GSK3 $\beta$  and drug activity in health and disease.



## Introduction

Glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) is a serine-threonine kinase that was first associated with control of energy homeostasis<sup>1-3</sup>; it is now also known for its prominent role in developmental processes including neurogenesis, neuronal polarization and axon growth during brain morphogenesis<sup>4</sup>.

The constitutively active form of GSK3 $\beta$  inhibits several signaling pathways, for example through phosphorylation of transcription factors<sup>1, 5</sup>. These signaling pathways are generally activated through phosphorylation of GSK3 $\beta$  at serine 9 which inhibits GSK3 $\beta$ 's enzymatic activity<sup>6</sup>. Prominent kinases phosphorylating serine 9 of GSK3 $\beta$  are Akt, also known as protein kinase B, protein kinase A and protein kinase C<sup>7-9</sup>. Cyclin-dependent kinase 5 (CDK5) increases phosphorylation of GSK3 $\beta$  apparently in an indirect manner by inhibiting protein phosphatase 2A and protein phosphatase 1<sup>10, 11</sup>.

Several lines of evidence suggest a key role of GSK3 $\beta$  activity in the development as well as treatment of major depressive disorder. Increased activity of GSK3 $\beta$ , along with decreased activity of Akt, has been found post mortem in the ventral prefrontal cortex of individuals with major depression<sup>12</sup>. In mice, decreasing the levels of GSK3 $\beta$  by deleting one allele of the *gsk3 $\beta$*  gene resulted in decreased depressive-like behavior<sup>13, 14</sup>. Similarly, reduction of GSK3 $\beta$  levels by injection of lentivirus expressing small hairpin RNA targeting GSK3 $\beta$  into the hippocampal dentate gyrus produced antidepressant-like effects in chronically stressed mice<sup>15</sup>. On the other hand, increasing the levels of GSK3 $\beta$  in mice by knock-in resulted in increased depressive-like behavior<sup>16</sup>.

GSK3 $\beta$  can be targeted by lithium and by antidepressants which inhibit GSK3 $\beta$  activity<sup>17, 18</sup>. Lithium is a frequently prescribed mood stabilizer that also amplifies the effect of antidepressants<sup>19</sup>. Lithium inhibits GSK3 $\beta$  both directly and indirectly through phosphorylation at serine 9, while only an indirect mode of action has been reported for

antidepressants so far<sup>17, 20</sup>. Newly developed inhibitors of GSK3 $\beta$  also display antidepressant-like effects in mice<sup>21</sup>.

Hence, the question arises whether and how other factors known to influence and mediate antidepressant action may link to GSK3 $\beta$  as important relay linked to several upstream and downstream pathways<sup>4</sup>. FK506 binding protein 51 (FKBP51) has been genetically associated with antidepressant response in several studies<sup>22, 23</sup>. FKBP51 is one of the Hsp90 cochaperones and has originally been characterized as regulator of steroid receptor function, and in particular of the glucocorticoid receptor<sup>24, 25</sup>. More recently, FKBP51 has been discovered as inhibitor of Akt by recruitment of the phosphatase PHLPP<sup>26</sup>. Thus, the genetic link to antidepressant response in depressed patients and the effect on the GSK3 $\beta$ -inhibitory kinase Akt prompted us to test whether FKBP51 impacts on GSK3 $\beta$  activity, thereby modulating the actions of psychoactive drugs. We also included the close homologue FKBP52 it frequently acts as functional antagonist of FKBP51<sup>24, 26-29</sup>.

## Results

*FKBP51 binds to and inhibits GSK3 $\beta$ , thereby relieving the suppressive effect of GSK3 $\beta$  on downstream targets.*

To test the hypothesis that FKBP51 changes GSK3 $\beta$  activity, phosphorylation of GSK3 $\beta$  at serine 9 (pGSK3 $\beta$ ) was determined after transient overexpression of FKBP52, FKBP51 and a series of mutants of FKBP51 into HEK cells. FKBP51 caused increased pGSK3, while the closely related FKBP52 showed no effect (Fig. 1A, suppl. Fig. 1A). The N-terminal FK1 domain harboring the peptidylprolyl-isomerase (PPIase) activity was required for increasing pGSK3 $\beta$  levels, while the enzymatic PPIase activity *per se* was dispensable. The effect of FKBP51 on GSK3 $\beta$  was also independent of Hsp90 interaction (Fig. 1A, suppl. Fig. 1A).

All mutants that inhibited Akt1 (Gassen et al., submitted) also triggered the elevation of pGSK3 $\beta$  levels. Thus, the increase of pGSK3 $\beta$  levels by FKBP51 cannot be explained by the observation of FKBP51 inhibiting Akt1 kinase activity. Therefore, we first verified that indeed FKBP51 inhibited Akt1 by reducing pAkt1 levels also in the chosen cellular paradigm (suppl. Fig. 1B and D). We then evaluated whether FKBP51 may act directly on GSK3 $\beta$ . Co-immunoprecipitation revealed that FKBP51 binds to GSK3 $\beta$  (Fig. 1B). FKBP51 constructs lacking the N-terminal FK1 domain did not interact with GSK3 $\beta$ , corresponding to their inability to increase pGSK3 $\beta$  levels. Neither binding to Hsp90 nor PPIase activity of FKBP51 were required for interaction with GSK3 $\beta$ . FKBP52 also bound to GSK3 $\beta$ , indicating that binding alone is not sufficient to change the phosphorylation status of GSK3 $\beta$ .

To monitor effects of FKBP51 downstream of GSK3 $\beta$  we measured TCF/LEF driven reporter gene activity. Increasing the levels of FKBP51 enhanced TCF/LEF-dependent transcriptional activity, in contrast to FKBP52 and consistent with FKBP51's inhibitory action on GSK3 $\beta$  (Fig. 1C, suppl. Figs 1 B and C). Further, we compared the effects of FKBP51 and FKBP52 on the GSK3 $\beta$  targets tau and  $\beta$ -catenin. The dose-dependent increase of pGSK3 $\beta$  by

FKBP51 was accompanied by decreased phosphorylation of tau (serines 396 and 622) and by increased levels of GSK3 $\beta$ -controlled  $\beta$ -catenin (Figs. 1 D-F, suppl. Figs. 1 B and C). The stark difference between FKBP52 and FKBP51 in affecting GSK3 $\beta$  phosphorylation was also reflected on the level of GSK3 $\beta$  targets.

#### *Change of GSK3 $\beta$ phosphorylation by CDK5 depends on FKBP51*

Since the inhibitory effect of FKBP51 on Akt cannot explain the observed FKBP51-induced inhibition of GSK3 $\beta$ , we tested whether FKBP51 influences the ability of CDK5 to enhance phosphorylation of GSK3 $\beta$ . We found that FKBP51 interacts with CDK5 (Fig. 2A). We next assessed whether pGSK3 $\beta$  levels depend on CDK5 in cells differing in their FKBP51 status: we made use of the CDK5 inhibitor roscovitine in wild-type (FKBP51<sup>+/+</sup>) mouse embryonic fibroblast cells (MEFs), FKBP51<sup>-/-</sup> MEFs and FKBP51<sup>-/-</sup> MEFs reconstituted by ectopically expressed FKBP51. Phosphorylation of GSK3 $\beta$  was dependent on CDK5 in all three cell systems, as indicated by reduced pGSK3 $\beta$  levels upon application of the CDK5 inhibitor (Fig. 2B). Ectopic expression of CDK5 increased pGSK3 $\beta$  levels in FKBP<sup>+/+</sup> cells, but not in FKBP<sup>-/-</sup> cells; reconstituting FKBP51 in FKBP51<sup>-/-</sup> cells by ectopic expression largely restored the effect of CDK5 on pGSK3 $\beta$  levels (Fig. 2B). This supports the conclusion that FKBP51 promotes phosphorylation of GSK3 $\beta$  through CDK5.

#### *FKBP51 amplifies the effects of lithium and antidepressants on GSK3 $\beta$ and downstream targets*

GSK3 $\beta$  is a target of the mood stabilizer lithium and of several antidepressants<sup>17, 18</sup>. Therefore, we tested whether FKBP51 influences the effects of psycho-active drugs on GSK3 $\beta$  and its downstream targets. Reporter gene assays in rat primary cortical astrocytes evidenced a dose-dependent increase of TCF/LEF activity upon exposure to lithium (Fig. 3A).

Coexpression of FKBP51 greatly amplified the effect of lithium, while FKBP52 had no effect. FKBP51 also enhanced the effects of lithium on TCF/LEF in rat primary cortical neurons (Fig. 3A). A synergistic effect on TCF/LEF-driven reporter activity was also observed in cortical astrocytes and neurons for FKBP51 in combination with the antidepressant paroxetine (PAR), and to a somewhat lesser extent for FKBP51 and valproic acid (VPA) (Fig. 3B).

To test whether the observed synergy between FKBP51 and lithium, PAR and VPA on TCF/LEF depends on GSK3 $\beta$ , we compared the effects in GSK3 $\beta^{+/+}$  and GSK3 $\beta^{-/-}$  MEFs. In GSK3 $\beta^{-/-}$  MEFs, TCF/LEF activity was higher than in GSK3 $\beta^{+/+}$  cells and did not respond to lithium, PAR, VPA or FKBP51 (Fig. 4A). However, in GSK3 $\beta^{+/+}$  MEFs, coexpression of FKBP51 enhanced the stimulatory effect of lithium and PAR on TCF/LEF-dependent transcription. This effect was much less pronounced in the case of VPA treatment. The drugs also elevated the levels of pGSK3 $\beta$  (Fig. 4B).

To further substantiate the conclusion that the action of psycho-active drugs depend on FKBP51, we made use of the FKBP51 $^{+/+}$  and FKBP51 $^{-/-}$  MEFs. Lithium and PAR increased pGSK3 $\beta$  in FKBP51 $^{+/+}$ , but not in FKBP51 $^{-/-}$  MEFs (Figs. 4C and D). In contrast, VPA increased pGSK3 $\beta$  irrespectively of the FKBP51 gene status (Figs. 4C and D). Reporter gene assays measuring TCF/LEF-dependent transcription showed similar TCF/LEF activity in untreated FKBP51 $^{+/+}$  and FKBP51 $^{-/-}$  MEFs. However, only FKBP51 $^{+/+}$  MEFs displayed a stimulation of TCF/LEF upon exposure to lithium and PAR, while VPA activated TCF/LEF irrespectively of the FKBP51 status (Fig. 4E).

To further characterize the synergism of FKBP51 and psycho-active drugs on GSK3 $\beta$  we tested whether lithium alters binding of FKBP51 to GSK3 $\beta$ . Treatment of HEK cells with lithium increased the interaction between FKBP51 and GSK3 $\beta$ , while PAR had only a minor and VPA no effect (Fig. 4F). Addition of lithium to cell extracts in the coimmunoprecipitation reaction also yielded a higher interaction of FKBP51 and GSK3 $\beta$ . The effects of lithium on

GSK3 $\beta$  phosphorylation have been found to be both directly and indirectly, while only indirect effects have been reported for antidepressants<sup>17, 20</sup>. Using kinase activity assays we confirmed a direct inhibition of GSK3 $\beta$  by lithium; we found in addition that paroxetine also inhibits GSK3 $\beta$  directly, albeit only to a minor extent at a concentration of 20  $\mu$ M (Figs. 4G and H).

#### *FKBP51 and psycho-active drugs in mice and human*

To test whether FKBP51 is linked to the actions of psychoactive drugs also *in vivo*, we treated FKBP51<sup>+/+</sup> and FKBP51<sup>-/-</sup> mice with lithium. The animals were assessed in the forced swim test, a widely used paradigm to measure depression-like behavior in mice. While the animals treated with vehicle did not display significant differences between FKBP51 genotypes, after lithium treatment, FKBP51<sup>+/+</sup> mice struggled longer and floated shorter times compared to FKBP51<sup>-/-</sup> mice (Fig. 5). In addition, lithium prolonged the latency to first floating in FKBP51<sup>+/+</sup>, but not in FKBP51<sup>-/-</sup> mice. We also determined the pGSK3 $\beta$ /GSK3 $\beta$  ratio in the hippocampus, prefrontal cortex and peripheral blood of lithium or PAR treated FKBP51<sup>+/+</sup> and FKBP51<sup>-/-</sup> mice. In all three tissues, both drugs increased pGSK3 $\beta$  in FKBP51<sup>+/+</sup>, but not in FKBP51<sup>-/-</sup> mice (Fig. 5).

To translate these findings to human, we first determined the expression levels of FKBP51, pGSK3 $\beta$ /GSK3 $\beta$  and beta-catenin in peripheral blood mononuclear lymphocytes (PBMCs) of healthy volunteers. A highly significant correlation between FKBP51 expression and pGSK3 $\beta$ /GSK3 $\beta$  was observed (Fig. 6). To evaluate the link to the action of psychoactive drugs, PBMCs were cultivated *ex vivo* and treated with lithium, PAR or VPA. To more closely mimic the clinically relevant *in vivo* conditions, drug concentrations were chosen according to the results of therapeutic drug monitoring in psychiatry<sup>30</sup>. The effect of lithium

and PAR on pGSK3 $\beta$ /GSK3 $\beta$  significantly correlated with the expression of FKBP51, while the effect of VPA was independent of the FKBP51 expression status (Fig. 6).

To further test the physiological relevance of the FKBP51-dependent actions of psychoactive drugs on GSK3 $\beta$  and downstream targets, we measured protein levels in PBMCs from 56 patients using a protein array and related them to clinical treatment response. We observed a significant negative correlation of the GSK3 $\beta$  target pTau (S622) with clinical treatment response (Fig. 7); a trend was received for pGSK3 $\beta$ /GSK3 $\beta$ .

## Discussion

The use of recent high-throughput technologies for genome-wide genotyping has spawned a wealth of association data in complex diseases; this progress entailed the need to apply complementary approaches to shed light on the identity and operation of the underlying signaling pathways and molecular networks<sup>31, 32</sup>. The results of this study suggest that the genetic association of FKBP51 with clinical response to antidepressant treatment is linked to FKBP51's action on GSK3 $\beta$ , which amplifies the impact of psycho-active drugs on this kinase.

Originally, we hypothesized that the known inhibitory effect of FKBP51 on Akt<sup>26</sup> increases GSK3 $\beta$  activity through decreased Akt-dependent phosphorylation of GSK3 $\beta$  at serine 9. It appears that the here presented direct interaction of FKBP51 with GSK3 $\beta$  is responsible for inhibition of GSK3 $\beta$  by increased phosphorylation, even though Akt is inhibited at the same time. Phosphorylation of Akt at serine 473 is required for enhanced kinase activity<sup>33, 34</sup> and has also been reported to enhance Akt-directed phosphorylation of GSK3 $\beta$ . However, others have found that phosphorylation of GSK3 $\beta$  by Akt is independent of serine 473 phosphorylation and only requires phosphorylation at threonine 308 for basal Akt activity<sup>35</sup>. Since FKBP51 only alters phosphorylation at serine 473, but not at threonine 308, there might be no consequence for GSK3 $\beta$  through FKBP51's effect on Akt in our experimental set-up.

The effect of FKBP51 on GSK3 $\beta$  likely involves CDK5, a regulator of GSK3 $\beta$  activity<sup>10, 11</sup>, which we report here as a newly identified FKBP51 interaction partner. These findings, together with FKBP51's impact on Akt and other signaling molecules lead us to propose that FKBP51 re-sets molecular pathways and networks by direct interaction with pivotal pathway elements. Since FKBP51 is a strongly stress-induced gene, this mechanism also efficiently links stress response to several pathways.



In contrast to FKBP51, the highly homologous FKBP52 displayed no effect on GSK3 $\beta$ , downstream targets and the effect of lithium. Differential outcomes between FKBP51 and FKBP52 are also known for their effects on corticosteroid receptors<sup>24, 25</sup>, microtubules<sup>27, 28</sup>, neurite outgrowth<sup>29</sup> and Akt<sup>26</sup> (Gassen et al. submitted). Functional domain mapping revealed the N-terminal FK1 domain as essential for the effect of FKBP51 on GSK3 $\beta$ ; the enzymatic activity of the PPIase situated in this region was not required. Similarly, the FK1 domain of FKBP51 was indispensable for its effect on the glucocorticoid receptor<sup>24, 25</sup>, as well as on Akt<sup>26</sup> (Gassen et al. submitted), but the PPIase activity was dispensable in both cases. Collectively, these data support the notion that the most important function of FKBP51's FK1 domain is not the isomerization of peptidylprolyl-bonds, but the assembly of protein complexes.

GSK3 $\beta$  has been extensively discussed as promising target of new psycho-active drugs<sup>36, 37</sup>. The results presented here add with FKBP51 a novel regulator of GSK3 $\beta$ , which at the same time has been linked to clinical AD response. The physiological relevance of this FKBP51-governed GSK3 $\beta$  pathway is further substantiated by *in vivo* experiments in mice and by providing strong evidence for a functional link of the GSK3 $\beta$  pathway to treatment response of depressed patients. The demonstration of the functionality of this pathway in a drug- and FKBP51-dependent manner in cultivated PBMCs opens the prospect for developing highly informative biomarkers. These may not only be derived from determination of pathway components in PBMCs from patients, but also from treatment of the *ex vivo* cultivated PBMCs with diverse antidepressants to monitor pathway response. These dynamic biomarkers would constitute a major advance towards implementing the concept of personalized medicine<sup>38</sup>.

**Experimental Procedures.** Details of the experimental methods can be found in the supplemental material.

## Figure Legends

**Figure 1.** *Functional interaction of FKBP51 with GSK3 $\beta$ .* **A**, constructs used in the interaction analyses and their effect on phosphorylation of GSK3 $\beta$  (S9). FK, FK506 binding domain; FL, full length. “PPIaseMut” denotes the F67V/D68V mutation abolishing PPIase activity; “Hsp90Mut” carries the K352A/R356A mutation disrupting interaction with Hsp90. HEK cells were transiently transfected with the plasmids encoding the listed FKBP constructs, all fused to a Flag-tag. The levels of GSK3 $\beta$  and pGSK3 $\beta$  were determined by Western blot and quantified (right panel). \*  $p < 0.05$ , \*\*  $p < 0.01$ , difference compared to FL Fkbp51. **B**, representative Western blots of the FKBP-GSK3 $\beta$  interaction analyses. Numbers on top of the panel correspond to the constructs listed in A. Plasmids were transfected into HEK cells and precipitation of protein complexes was performed using magnetic protein G beads conjugated to a Flag-antibody; (co)precipitated proteins were visualized by Western blot and quantified. Numbers at the bottom of the panel summarize the results of 3 independent experiments (3 technical replicates of the Western blots of each experiment) analyzing the interaction of the FKBP5s with GSK3 $\beta$ . **C**, The functional effect of FKBP-governed differential GSK3 $\beta$  phosphorylation was assessed by TCF/LEF-driven reporter gene analysis in HEK cells transiently overexpressing increasing amounts of full-length FKBP51 or 52. Expression efficiency was controlled by Western blot analysis (right panel). Relative reporter activity is given in arbitrary units reflecting the mean  $\pm$  SEM of three independent experiments performed in triplicate. **D-F**, HEK cells were transfected with either control plasmid or two different amounts of FKBP51 or FKBP52 expressing plasmid (1.5 or 3  $\mu$ g plasmid/  $10^7$  cells), and the protein parameters were determined by Western blot. Graphs display the difference of protein phosphorylation or abundance in comparison to vector control as the relative mean expression  $\pm$  SEM of 3 different experiments. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

**Figure 2.** *FKBP51 interacts with CDK5 that changes GSK3 $\beta$  phosphorylation.* **A**, HEK cells were transfected with FLAG-tagged FKBP51 expressing plasmid or control vector and interaction of FKBP51 and CDK5 was evaluated by co-immunoprecipitation as indicated. Representative Western blot is shown. **B**, comparison of pGSK3 $\beta$  in FKBP51<sup>+/+</sup>, FKBP51<sup>-/-</sup>, and FKBP51<sup>-/-</sup> MEFs reconstituted by ectopic expression of FKBP51. Cells were (co)transfected with a CDK5 expressing plasmid or treated with the CDK5 inhibitor Roscovitine (0.3  $\mu$ M) as indicated. Phosphorylation of GSK3 $\beta$  was determined by Western blot. Representative blot and quantification of pGSK3b as mean + SEM of three different experiments (each analyzed in three technical replicates) are displayed. \* p<0.05; \*\* p<0.01; \*\*\* p<0.001.

**Figure 3.** *FKBP51 amplifies the effects of lithium and paroxetine on GSK3 $\beta$ .* TCF/LEF reporter gene assays were used as read out of the ability of GSK3 $\beta$  to suppress downstream targets. Primary cortical astrocytes (A and B) or primary cortical neurons (A and B) were transfected with reporter and FKBP51 or FKBP52 expressing plasmids and treated with the drugs as indicated for 48h. Reporter activity in vehicle-treated and vector-transfected cells was set to 1. Data represent results from 3 independent experiments performed in triplicate. Drug concentrations were: PAR 10 $\mu$ M, VPA 10mM, Li 10mM unless indicated otherwise. \* p<0.05; \*\* p<0.01; \*\*\* p<0.001.

**Figure 4.** *FKBP51-dependent and –independent effects of lithium and paroxetine on GSK3 $\beta$ .* **A,E**, GSK3 $\beta$ <sup>-/-</sup> and GSK3 $\beta$ <sup>+/+</sup> MEFS (A) or FKBP51<sup>+/+</sup> and FKBP51<sup>-/-</sup> MEFs (E) were transfected with TCF/LEF-reporter and FKBP51 or FKBP52 expressing plasmids and treated with the drugs as indicated for 48h. Reporter activity in vehicle-treated and vector-transfected

cells was set to 1. **B-D**, the ratios of pGSK3 $\beta$  to GSK3 $\beta$  were determined in the indicated MEFs after drug treatment. Drug concentrations in A-E: PAR 10 $\mu$ M, VPA 10mM, Li 10mM. **F**, interaction analysis of FKBP51 and GSK3 $\beta$  by coimmunoprecipitation. HEK cells were transfected with FKBP51-FLAG and GSK3 $\beta$  expressing plasmids, treated with drugs as indicated for 48h, and precipitation of protein complexes was performed using magnetic protein G beads conjugated to a Flag-antibody; (co)precipitated proteins were visualized by Western blot and quantified. **G, H**, effect of lithium and PAR on kinase activity of purified GSK3 $\beta$ . GSK3 $\beta$  was exposed to drug as indicated and kinase reaction was started by adding biotinylated substrate and ATP. Substrate phosphorylation was determined by mass spectrometry. All data represent results from 3 independent experiments performed in triplicate. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

**Figure 5.** *FKBP51 determines the effect of lithium and PAR in mice.* FKBP51<sup>-/-</sup> and FKBP51<sup>+/+</sup> mice were treated with lithium or vehicle and subjected to the forced swim test (N = 7-9 per group). Graphs show the times of struggling, swimming, floating and latency to first floating. For determining FKBP51-dependency of drug effects on pGSK3 $\beta$  in mice, FKBP51<sup>-/-</sup> and FKBP51<sup>+/+</sup> mice were treated with lithium (50 mg kg<sup>-1</sup>), PAR (10 mg kg<sup>-1</sup>) or the respective vehicle and sacrificed 45 mins later. Levels of the indicated proteins were determined by Western blotting in extracts of the hippocampus, prefrontal cortex and blood from 9-10 animals in technical triplicates. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

**Figure 6.** *Expression of FKBP51 in human peripheral blood lymphocytes correlates with pGSK3 $\beta$  and the effects of lithium and PAR.* PBMCs were collected from healthy male volunteers (N=20) and protein levels were determined in cell extracts by Western blotting. PBMCs were also cultivated *ex vivo* and treated with lithium (1mM), PAR (0.365  $\mu$ M), VPA

(1mM) or vehicle for 48 h and protein levels were determined in cell extracts by Western blotting. Change of pGSK3 $\beta$ /GSK3 $\beta$  was calculated from the comparison of drug treated cells with vehicle treated cells. Each dot represents the levels of the respective proteins in PBMCs from one individual. Average expression levels were set to 1.

**Figure 7.** *Correlation analysis of pGSK3 $\beta$  and its target tau with clinical treatment response.*

PBMCs were collected from depressive patients at the day of admission to the clinic (N=54). Protein concentrations in extracts were determined on a protein array and correlated to the change in Hamilton Depression Score.

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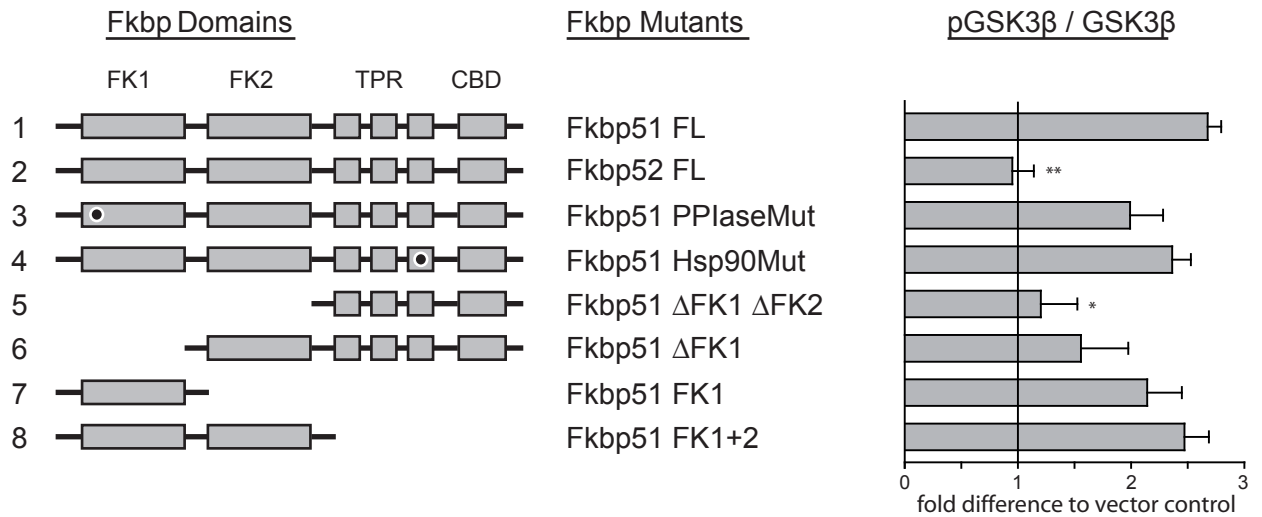
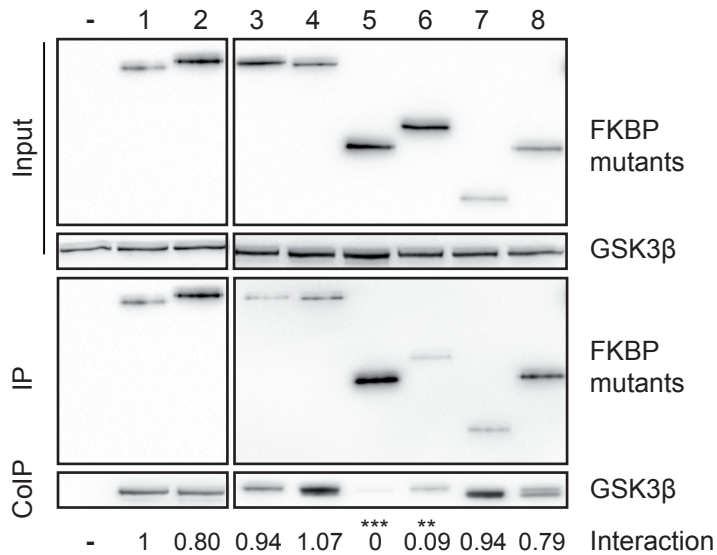
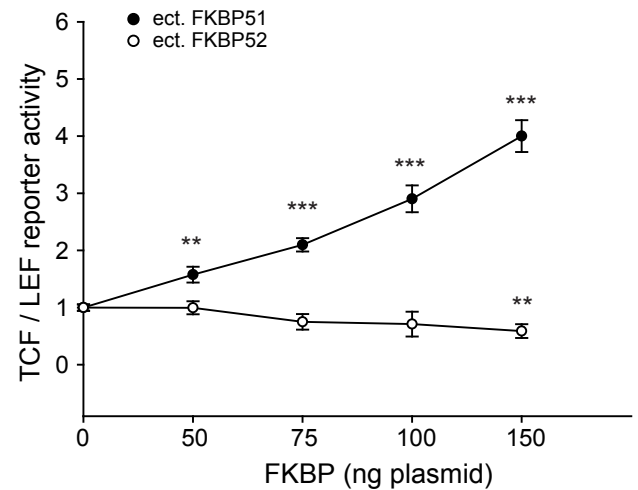
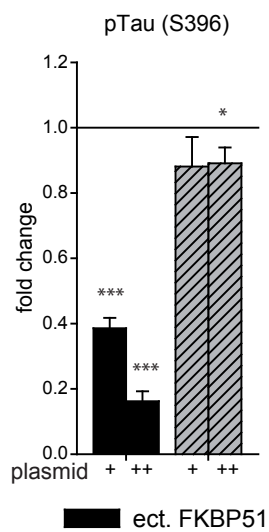
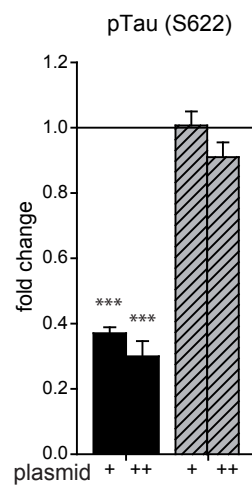
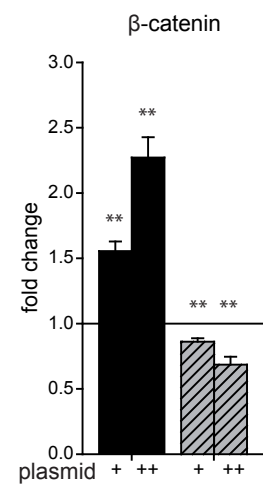
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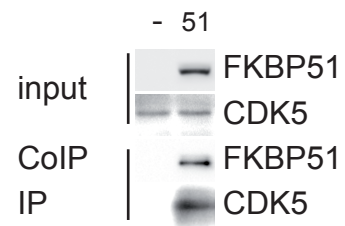
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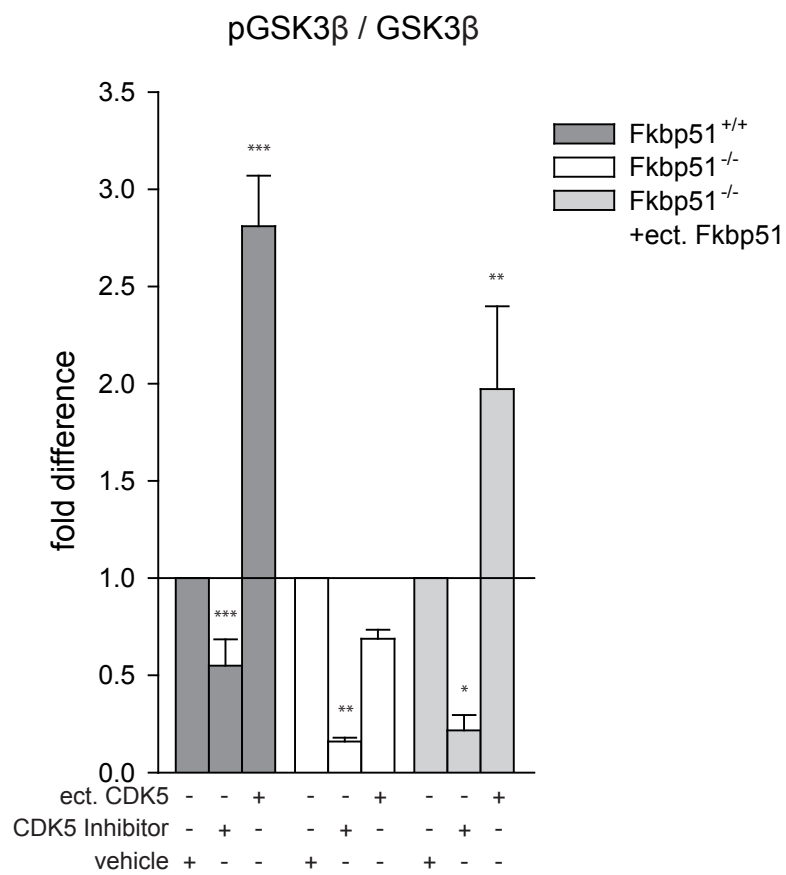
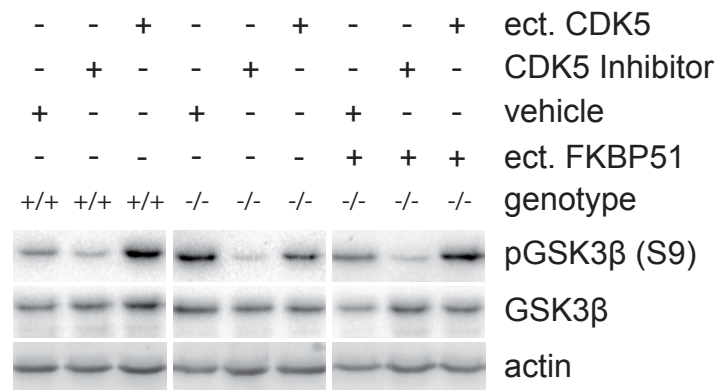
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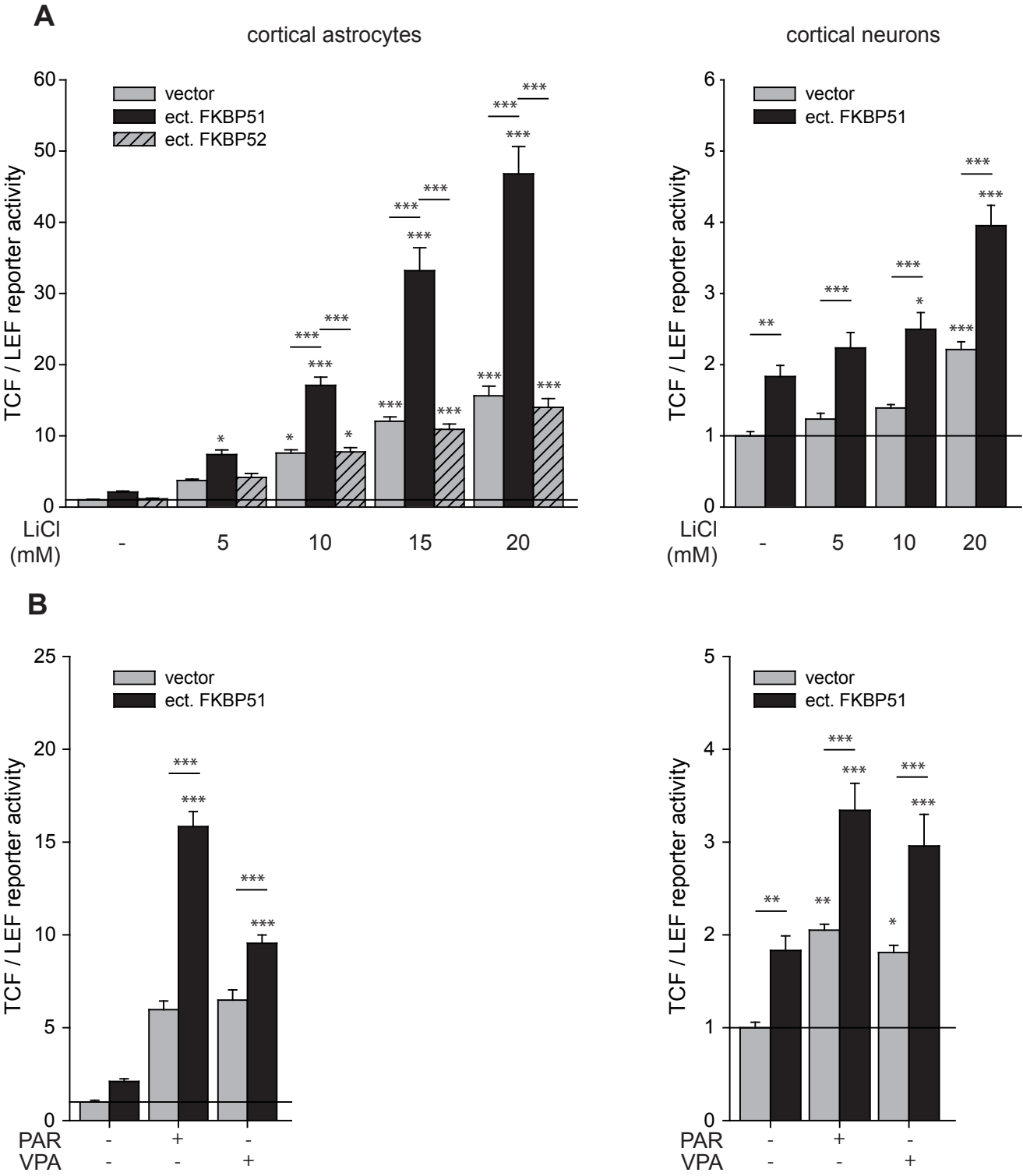
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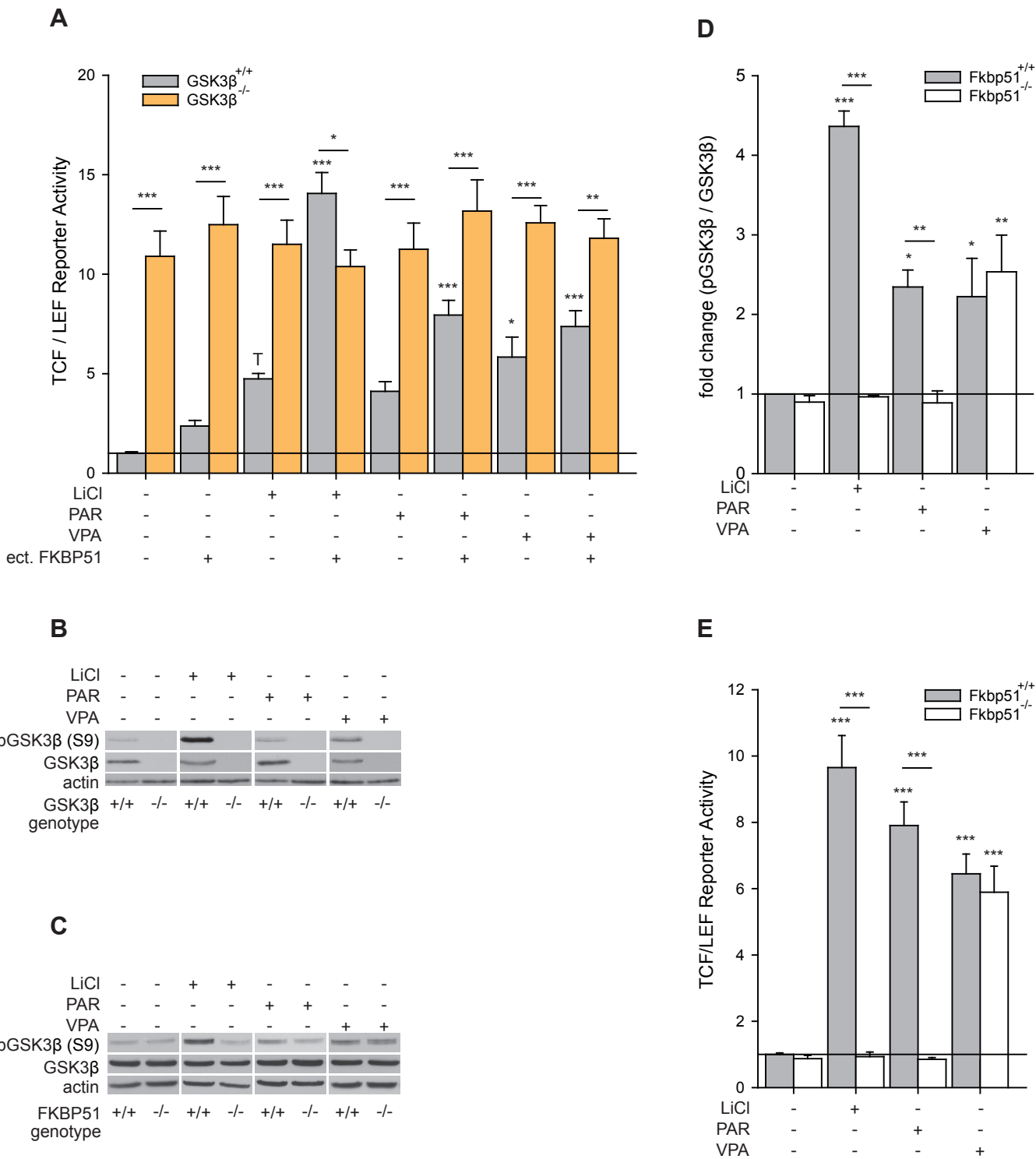
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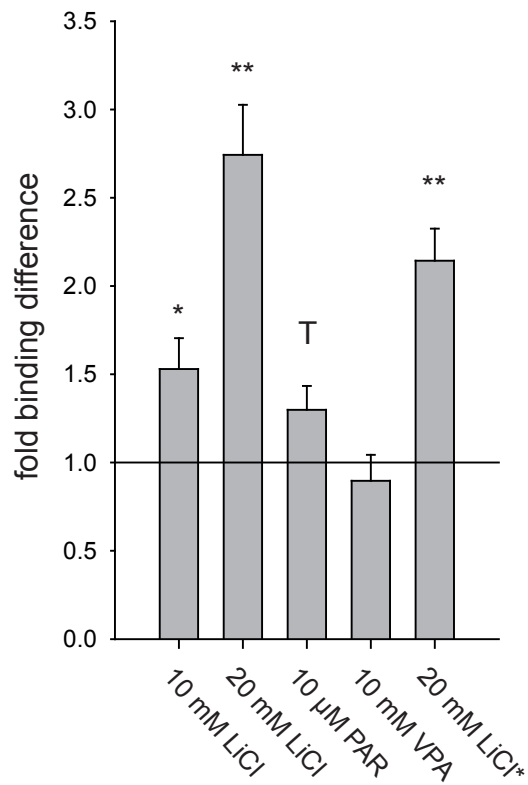
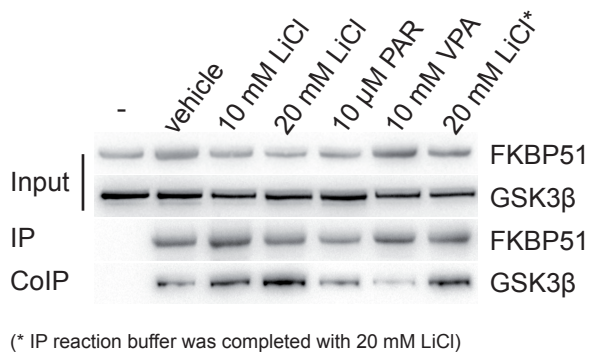
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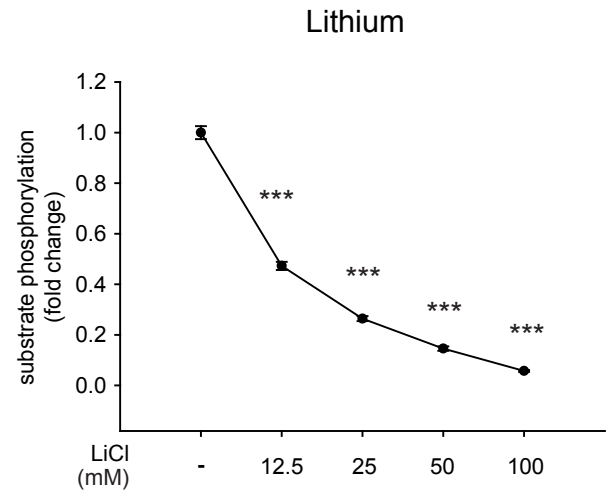




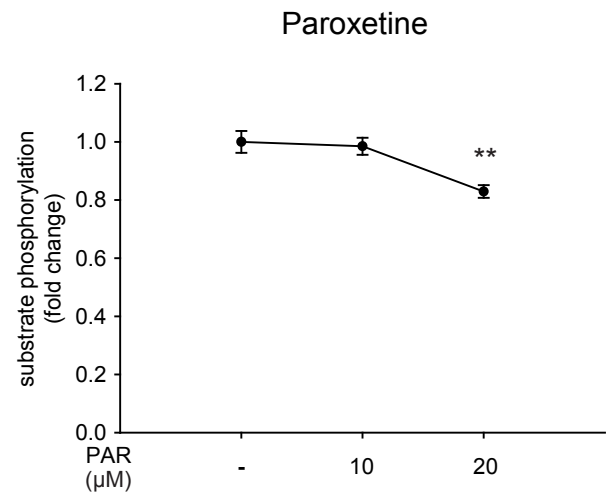
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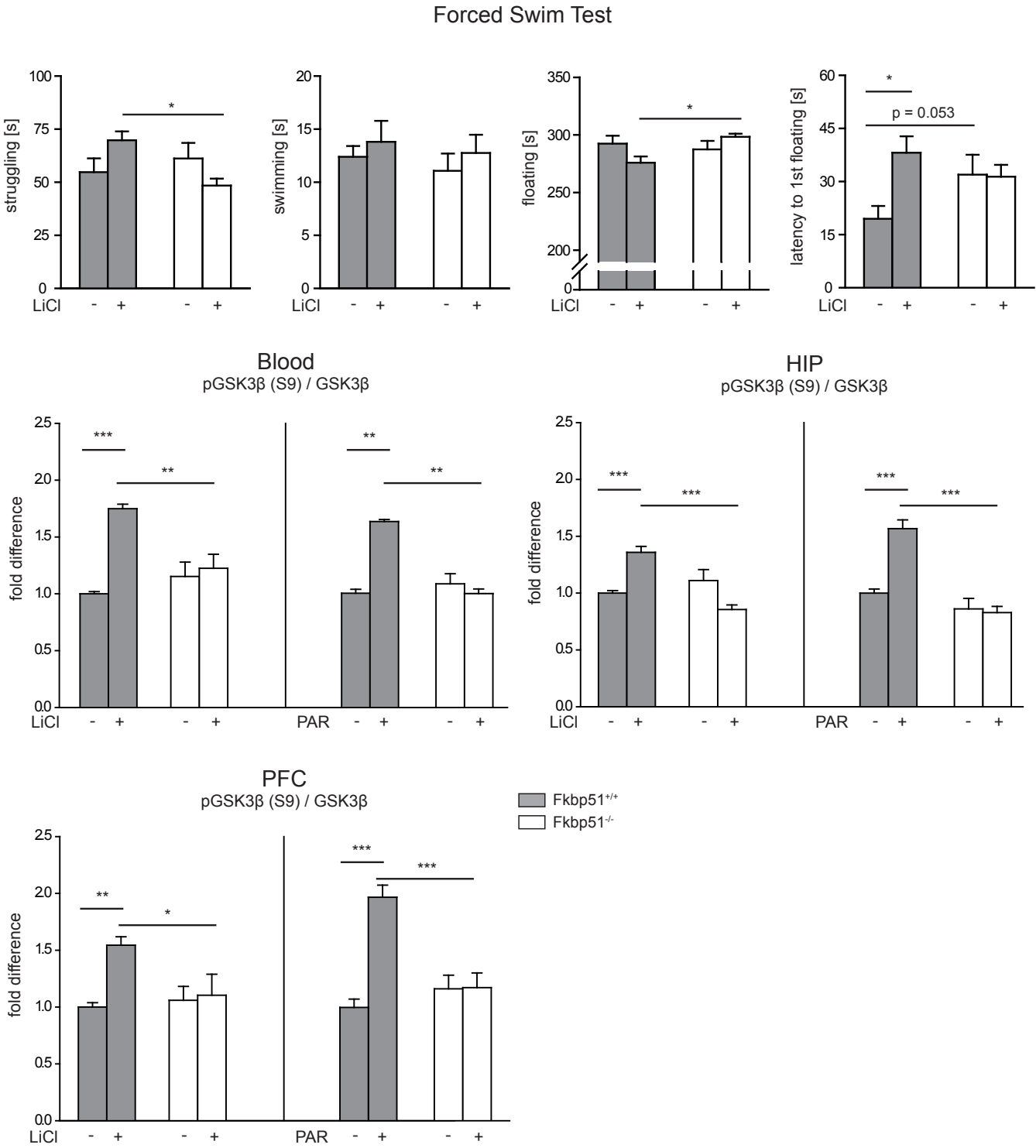
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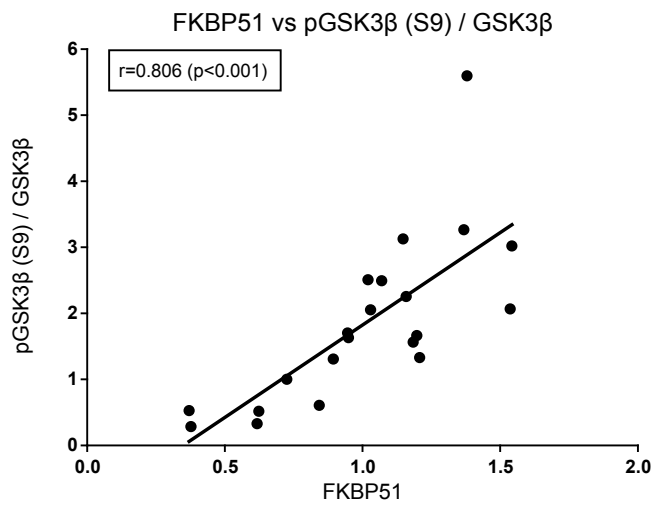
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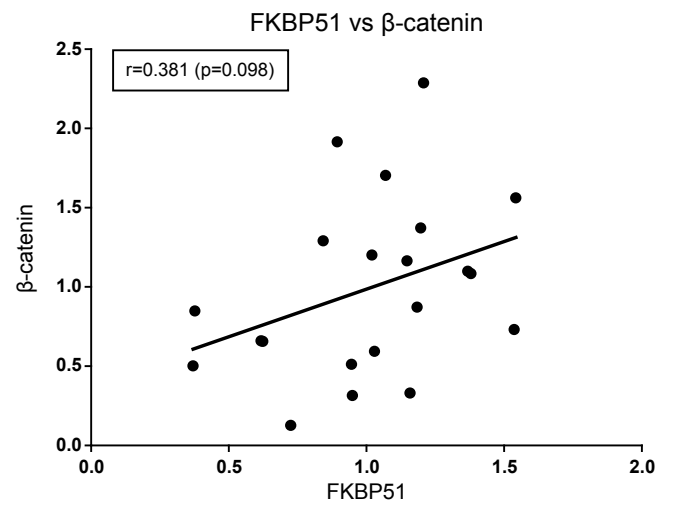




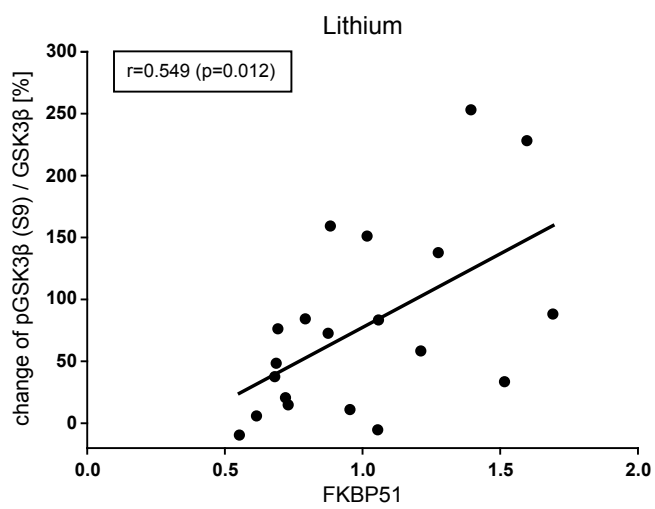
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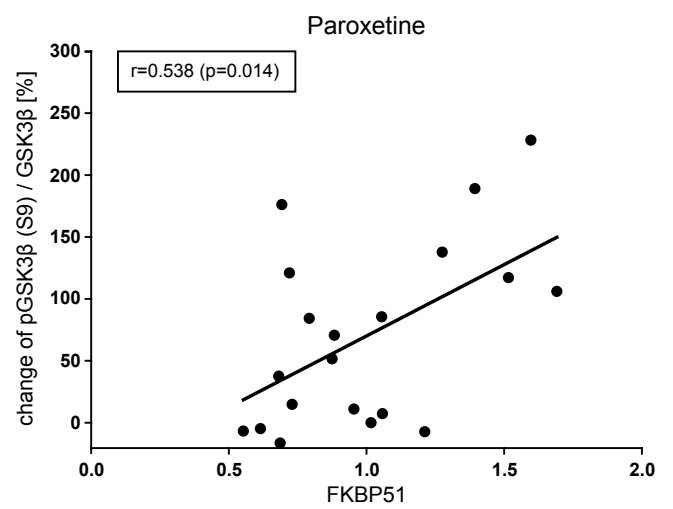
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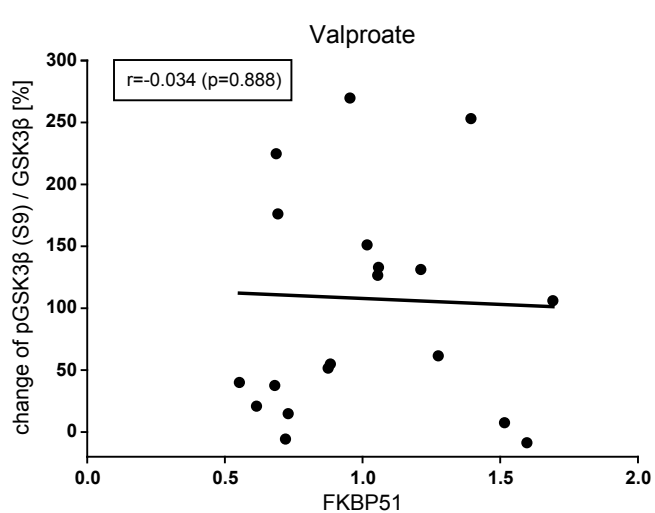
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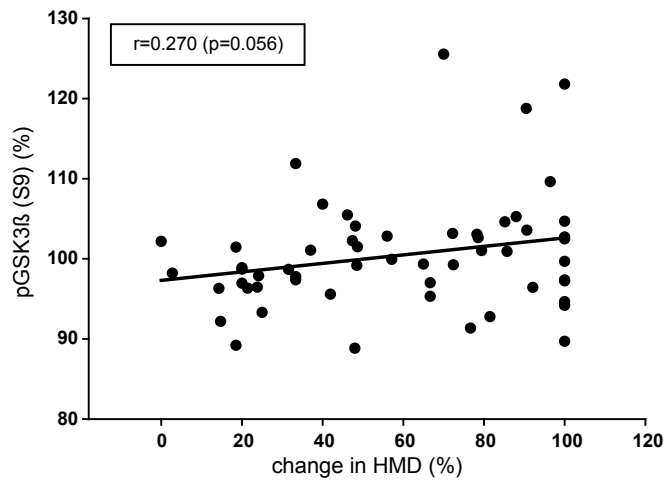
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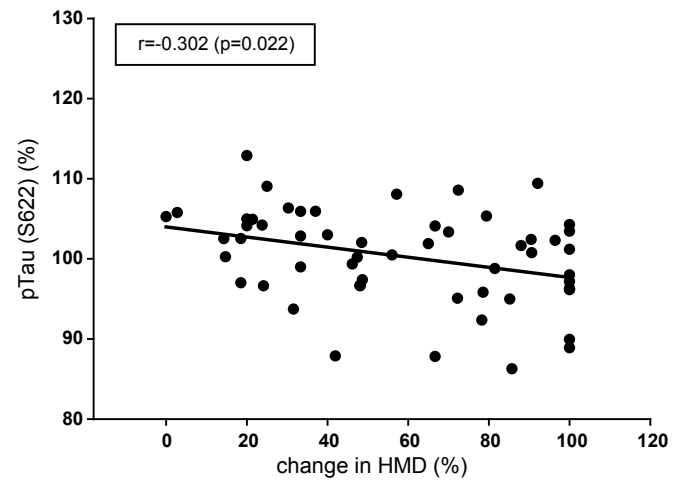
E



**A**



**B**



## Supplemental Material to Gassen et al.,

### FKBP51 inhibits GSK3 $\beta$ and amplifies the effects of distinct psychoactive drugs

#### LEGENDS TO SUPPLEMENTAL FIGURES

**Supplementary Figure 1.** *FKBP51 dependency of GSK3 $\beta$  pathways.* The impact of FKBP51 expression on pGSK3 $\beta$ , phosphorylation of the GSK3 $\beta$  target tau and level of the GSK3 $\beta$  target  $\beta$ -catenin. HEK cells were transfected with either control plasmid or two different amounts of FKBP51 or FKBP52 expressing plasmid (1.5 or 3  $\mu$ g plasmid/  $10^7$  cells), and the protein parameters were determined by Western blot. **A**, representative Western blots; **B-E**, graphs display the difference of protein phosphorylation or abundance in comparison to vector control as the relative mean expression + SEM of 3 different experiments. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

#### MATERIALS AND METHODS

**Cell lines** - Human embryonic kidney cells (HEK-293, ATCC CRL-1573) and mouse embryonic fibroblasts (MEFs) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FCS and 100 units/ml penicillin and streptomycin, respectively. FKBP51<sup>-/-</sup>, GSK3 $\beta$ <sup>-/-</sup> (kind gift of James Woodgett, Ontario Cancer Institute, Toronto, Canada) and Akt1/2<sup>-/-</sup> MEFs (kind gift of Nissim Hay, University of Illinois, Chicago, Illinois) have been described before<sup>1-3</sup>.

**Primary cultures of rat astrocytes and neurons** - Enriched astroglial cultures were prepared from postnatal day 1 rat pups (Sprague-Dawley, Charles River, Sulzfeld, Germany) and handled as described<sup>4-6</sup>.

**Transfection in MEF Fkbp51<sup>-/-</sup> cells, rat primary astrocytes and neurons** – Afore detached MEF cells ( $2 \times 10^6$ ) were re-suspended in 100  $\mu$ l transfection buffer (50 mM HEPES pH 7.3, 90 mM NaCl, 5 mM KCl, 0.15 mM CaCl<sub>2</sub>)<sup>7</sup>. A maximal amount of 5  $\mu$ g plasmid DNA was added to the cell suspension, and electroporation was carried out using the Amaxa Nucleofactor system (program # T-020). For neurons, the rat neuron nucleofactor kit was used (#VDP-1003). Cells were re-plated at a density of  $10^5/\text{cm}^2$  and further processed for Western blot analysis.

**Preparation of human PBMCs** – Blood of healthy male volunteers was collected via venipuncture, diluted with PBS and carefully loaded on Biocoll solution (1BioChrom AG, L6113) and centrifuged at  $800 \times g$  for 20 min (brakeless running down). PBMCs were enriched by selecting the interphase of the Biocoll gradient. PBMCs of the interphase were washed two times with ice-cold PBS. PBMCs were re-suspended in RPMI and plated at  $4 \times 10^5/\text{cm}^2$ . After recovery for 6 h, cells were treated with either, 190  $\mu$ M (8  $\mu$ g ml<sup>-1</sup>) lithium chloride, 694  $\mu$ M (100 ng ml<sup>-1</sup>) VPA, 365 nM (120 ng ml<sup>-1</sup>) PAR. Concentrations had been chosen to match therapeutic concentrations in the serum according to the consensus guidelines for therapeutic drug monitoring in psychiatry<sup>8</sup>.

**Plasmids** – FKBP51 deletion mutants were constructed using the pRK5-FKBP51 plasmid as template<sup>9</sup>. PCR was performed as described before<sup>9</sup> with the following combination of

primers; dFK1, fwd5'-TCTCTCCACTCGAGATA TGAAAGGAGAGGATTTATTTG-3, rev 5'-CTCTTCTTGCGGCCGCTCACTTGTCA TCGTCGTCCTTG TAGTCTACGTGGCCCTCAGGTTTCTC-3'; dFK1+FK2, fwd5'-TCTCTCCA CTCGAGATA TGAAGGCCAAAGAATCCTGGG-3', rev 5'-CTCTTCTTGCGGCCGCTCACTTGT CATCGTCGTCCTTG TAGTCTACGTGGCCC TCAGGTTTCTC-3'; FK1+FK2, fwd5'-TTCTTTCCA CTCGAGATA TGA CTA CTG ATG AAG G-3', rev. 5'-CTCTTCTT GCGGCCGCTCACTTGT CATCGTCGTCCTTG TAGTCTTCGAAGCTCTTAAGTGTAAC-3'; FK2, fwd5'-TCTCTCCACTCGAGATA TGAAAGGAGAGGATTTATTTG-3, rev 5'-CTCTTCTT GCGGCCGCTCACTTGT CATCGTCGTCCTTG TAGTCTTCGAAGCTCTTAAGTGTAAC-3'. All clones were checked by Sanger sequencing.

Plasmids expressing TCF/LEF-Luc, CDK5-HA and FKBP52 have been described<sup>9-11</sup>.

**Reporter gene assays** - Reporter gene experiments were carried out in 96-well formats.  $2 \times 10^6$  cells were transfected with 1000 ng TCF/LEF-Luc, 100 ng pCMV-Gaussia-Luc and 1000 ng pRK5-FKBP51/52-Flag -plasmid using Amaxa Nucleofactor system as described above. Drug treatment was usually for 48h as specified in the figure legends. To determine Firefly luciferase activity, cells were lysed in 50  $\mu$ L passive lysis buffer (0.1 M KPO<sub>4</sub> pH 7.8, 0.2% Triton); addition of 50  $\mu$ L of luciferase reaction buffer to a fraction of 10  $\mu$ L of cell lysate (33 mM KHPO<sub>4</sub>, pH 7.8, 1.7 mM ATP, 3.3 mM MgCl<sub>2</sub>, and 13 mM luciferin) and luminometric readings were performed with an automatic counter (Tristar, Berthold). Activity of secretory Gaussia luciferase<sup>12</sup> was measured after addition of 50  $\mu$ L of substrate/buffer (1.1 M NaCl, 2.2 mM EDTA, 0.22 M KPO<sub>4</sub> pH 5.1, 0.44 mg ml<sup>-1</sup> BSA, and 0.5 mg ml<sup>-1</sup> coelenterazine).

**Co-Immunoprecipitation(CoIP)** - CoIP of FLAG-tagged FKBP51/52 mutants with endogenous GSK3 $\beta$  or endogenous CDK5 were performed in HEK-293 cells.  $5 \times 10^6$  cells were electroporated with 5  $\mu$ g of the respective expression plasmids using a GenePulser (Bio-Rad, USA) at 350 V/700  $\mu$ F in 400  $\mu$ l of electroporation buffer (50 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, 20 mM KAc, pH 7.35, 25 mM MgSO<sub>4</sub>). After three days of cultivation in DMEM/10% FCS, cells were lysed in CoIP-buffer containing 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Igepal complemented with protease inhibitor cocktail (Sigma, P2714). This was followed by incubation on an overhead shaker for 20 min at 4 °C. The lysate was cleared by centrifugation, the protein concentration was determined, and 1.2 mg of lysate was incubated with 2.5  $\mu$ g FLAG antibody overnight at 4 °C. 20  $\mu$ l of BSA-blocked Protein G Dynabeads (Invitrogen, 100-03D) were added to the lysate–antibody mix followed by 3 h incubation at 4 °C. The beads were washed 3 times with PBS and protein-antibody complexes were eluted with 100  $\mu$ l of 1 $\times$  FLAG-peptide solution (Sigma, 100–200  $\mu$ g ml<sup>-1</sup>, F3290) in CoIP buffer for 30 min at 4 °C. 5–15  $\mu$ g of the cell lysates or 2.5  $\mu$ l of the immunoprecipitates were separated by SDS-PAGE.

**Western Blot analysis** – Protein extracts were obtained by lysing cells in 62.5 mM Tris, 2% SDS and 10% sucrose, supplemented with protease (Sigma , P2714) and phosphatase (Roche, 04906837001) inhibitor cocktail. Samples were sonicated and heated at 95 °C for 5 min. Proteins were separated by SDS-PAGE and electro-transferred onto nitrocellulose membranes. Blots were placed in Tris-buffered saline, supplemented with 0.05% Tween (Sigma, P2287) and 5% non-fat milk for 1 h at room temperature and then incubated with primary antibody (diluted in TBS/0.05% Tween) overnight at 4 °C. The following primary antibodies were used: Beclin1 (1:1000, Cell Signaling, #3495), GSK3 $\beta$  (1:1000, Cell Signaling, #9315), LC3B-I/II (1:1000, Cell Signaling, #2775), FLAG (1:7000, Rockland, 600-401-383), HA-HRP conjugated (1:25000, Roche, 11667475001), FKBP51 (1:1000,

Bethyl, A301-430A ), FKBP52 (1:2000, Bethyl, A301-427A), Akt (1:1000, Cell Signaling, #4691), pAkt (1:1000, Ser472, T308, 1:1000, Cell Signaling, #4058, #9275), pGSK3 $\beta$  (1:1000, Cell Signaling, #9323), PI3K Class III (Vps34, 1:1000, Cell Signaling, #4263), ptau (SS396; Abcam, #ab109390), ptau (S622; SCBT, #sc-16938), Actin (1:5000, Santa Cruz Biotechnologies, sc-1616).

Subsequently, blots were washed and probed with the respective horseradish peroxidase- or fluorophore-conjugated secondary antibody for 1 h at room temperature. The immuno-reactive bands were visualized either using ECL detection reagent (Millipore, Billerica, MA, USA, WBKL0500) or directly by excitation of the respective fluorophore. Determination of the band intensities were performed with BioRad, ChemiDoc MP, or X-ray-films

**Animals and animal housing** – The Fkbp51 knockout (Fkbp51<sup>-/-</sup>) mouse line was previously generated<sup>1, 13</sup> and fully backcrossed to C57/Bl6. Genotypes were verified by PCR of tail DNA. Only male mice were used for the experiment, obtained from heterozygous breeding pairs. Animals were between 10 and 16 weeks old at the start of the experiment. Mice were held under standard conditions (12L: 12D light cycle, lights on at 08:00 am, temperature 23 $\pm$ 2°C), were singly housed and acclimated to the room for one week before the beginning of the experiments. Food (Altromin 1314, Altromin GmbH, Germany) and tap water were available *ad libitum*.

All experiments were carried out in the animal facilities of the Max Planck Institute of Psychiatry in Munich, Germany. The experiments were carried out in accordance with the European Communities' Council Directive 86/609/EEC. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria, Germany.



**Animal treatments and behavioral experiments** – All mice were injected intraperitoneally either with a single dose of lithium (85 mg kg<sup>-1</sup>; dissolved in saline; Sigma-Aldrich, Germany), paroxetine (10 mg kg<sup>-1</sup>; dissolved in saline; Sigma-Aldrich, Germany) or saline vehicle solution. The injection volume was 5 ml kg<sup>-1</sup> body weight. 45 min after the injection, two subgroups of mice (first group: WT vehicle, n = 10; Fkbp51<sup>-/-</sup> vehicle, n = 9; WT lithium, n = 10; Fkbp51<sup>-/-</sup> lithium, n = 10; WT; second group: WT vehicle, n = 10; Fkbp51<sup>-/-</sup> vehicle, n = 9; WT paroxetine, n = 10; Fkbp51<sup>-/-</sup> paroxetine, n = 10; WT) were sacrificed by decapitation following quick anesthesia by isoflurane. Trunk blood was collected in 1.5 ml EDTA-coated microcentrifuge tubes (KabeLabortechnik, Germany) and kept on ice until further processing. Furthermore, brains were removed; hippocampus and prefrontal cortex were extracted and stored on dry ice until further processing.

Another subgroup of mice (WT vehicle, n = 8; Fkbp51<sup>-/-</sup> vehicle, n = 8; WT lithium, n = 9; Fkbp51<sup>-/-</sup> lithium, n = 7) was subjected to a forced swim test (FST) 45 min after the injection. The FST was carried out between 9:00 am and 10:30 am in the same room in which the mice were housed and was analyzed using an automated video-tracking system (Anymaze 4.20, Stoelting, IL; USA). In the FST, each mouse was put into a 2 l glass beaker (diameter: 13 cm, height: 24 cm) filled with tap water (21 ± 1 °C) to a height of 15 cm, so that the mouse could not touch the bottom with its hind paws or tail. Testing duration was 6 min. Time spent immobile and time spent struggling was scored by an experienced observer, blind to genotype or treatment of the animals.

### **GSK3β kinase activity**

Determination of GSK3b kinase activity in the presence of lithium, paroxetine or vehicle was performed following a published protocol<sup>14</sup>.

### **Statistical analysis**

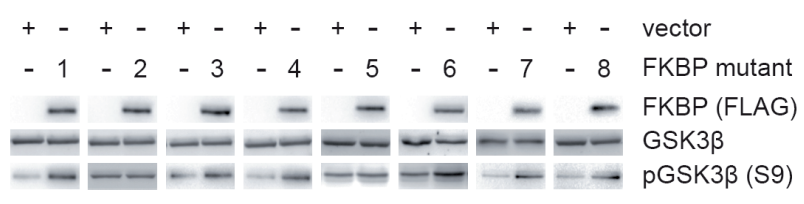
All statistical analyses were performed with SPSS 16.0. When two groups were compared, the student's *t*-test was applied. For three or more group comparisons, one-way or two-way analysis of variance (ANOVA) was performed, followed by Tukey's post-hoc test, as appropriate. All ANOVA F and p values are reported in supplementary table 1; significant results of the contrast tests are indicated by asterisks in the graphs. Protein-protein associations were analyzed using the Pearson correlation coefficient. P values of less than 0.05 were considered significant.

## Reference List

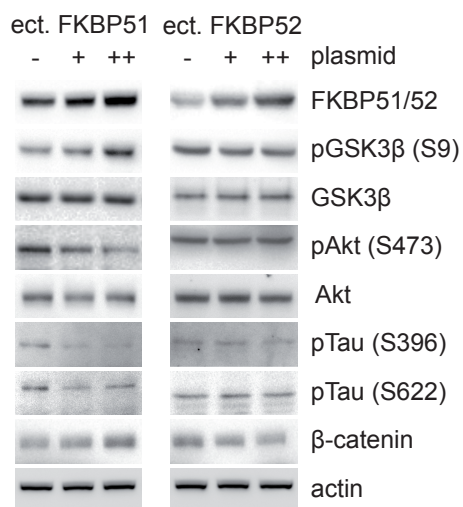
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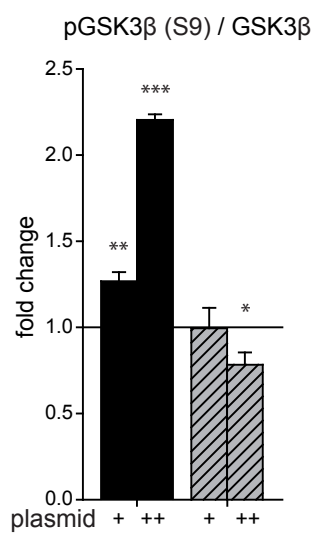
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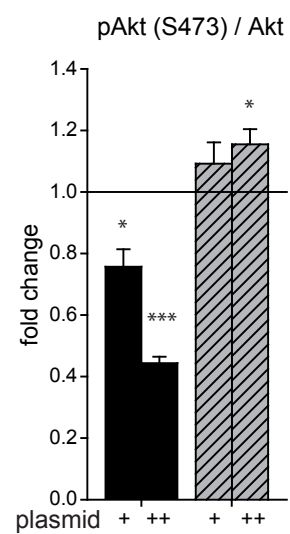
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■ ect. FKBP51

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## **FKBP51 shapes antidepressant actions**

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## Abstract

Clinical studies established a genetic link between the cochaperone FKBP51 and antidepressant (AD) response in mood disorders while the underlying mechanisms remain elusive. We provide first *in vitro* and *in vivo* evidence for a role of FKBP51 in priming Akt-dependent pathways for AD action on neuronal function and behavior. We found that inhibition of Akt by FKBP51 is followed by Akt-downstream cascades triggering activation of FoxO3a, inhibition of mTOR, and induction of autophagy markers. ADs modulate these pathways in a similar fashion; intriguingly, these AD effects are blunted in *Fkbp51*<sup>-/-</sup> cells and mice. Likewise, ADs' impact on neuronal function and on behavior is abolished in *Fkbp51*<sup>-/-</sup> mice. In human blood cells, the FKBP51 expression status strongly correlates with the potential of ADs to act on the respective pathways. Together, these findings provide a functional link between ADs and FKBP51, the latter sensitizing the organism for AD actions.

## Introduction

FKBP51 (FK506 binding protein 51) is a regulator of the glucocorticoid receptor, and consequently of the stress hormone axis and stress physiology<sup>1-5</sup>. In addition, human genetic studies link FKBP51 to the antidepressant response rate<sup>6-9</sup>. Despite the intimate connection of stress physiology to pathophysiology and treatment of depression<sup>10, 11</sup>, the mechanistic role of FKBP51 for antidepressant response has remained enigmatic.

Convergent molecular pathways addressed by both FKBP51 and antidepressants could explain the impact of FKBP51 on antidepressant response; modulation of these pathways by FKBP51 could thus influence their reactivity towards antidepressants. The hypothesis that these pathways involve Akt and downstream autophagic events is derived from two observations: some antidepressants modulate Akt<sup>12, 13</sup> as well as autophagy<sup>14-17</sup> and FKBP51 inhibits Akt<sup>18</sup>. This protein kinase exhibits interdependency with mTOR (mammalian target of rapamycin) and thereby affects autophagy<sup>19-24</sup>. Very recently, also an mTOR-independent effect of Akt on autophagy has been found by direct phosphorylation of Beclin1<sup>25</sup>.

Autophagy is a conserved cellular degradation process ensuring continuous removal of damaged macromolecules and thus functional integrity of cells and tissues<sup>26</sup>. Different types of autophagy are described in the literature<sup>27</sup>. Macroautophagy involves the step-wise formation of an autophagosome engulfing damaged organelles or proteins and subsequent fusion with a lysosome yielding the autolysosome. This process is orchestrated by a series of autophagy-related genes (Atg) and is regulated by complex pathways, which are partly under control of the mTOR kinase<sup>28, 29</sup>. Corresponding to this multitude of involved factors, experimental evaluation of autophagy is usually based on the determination of several markers. These include, but are not limited to, lipidation of LC3B-I (yielding LC3B-II), Atg12, Beclin1 and Vps34 (class III phosphatidylinositol-3-kinase). Chaperone-mediated autophagy is a more recently characterized selective type of autophagy; distinct proteins are



identified by chaperones, directed to lysosomes, unfolded and translocated to the inside of lysosomes<sup>30</sup>.

As guardian of cellular homeostasis, autophagy is a pivotal process in a range of (patho-) physiological conditions including infectious diseases, cancer, and diabetes, and recently also neurotransmission and neurodegeneration<sup>31-34</sup>. Indirect evidence has been provided for a role of autophagy in depression: some antidepressants change the autophagic flux and the expression of autophagy markers<sup>14-17</sup>. Moreover, induction of autophagy by the mTOR inhibitor rapamycin was reported to exert antidepressant-like effects in mice<sup>35</sup>.

In this study, we characterized the physical and functional interaction of FKBP51 with Akt, tested whether this interaction impacts on downstream signaling events and markers of autophagy, and evaluated convergent, FKBP51-dependent molecular actions of antidepressants on signaling pathways, neuronal function and behavior in life cells, mice and humans.

## Results

### *Identification of FKBP51-Akt directed pathways and downstream autophagy*

As candidate mechanism for convergent actions of antidepressants and FKBP51, we first characterized the reported effect FKBP51 on the protein kinase Akt<sup>18</sup>. A series of FKBP51 deletion and point mutant constructs as well as the close homologue FKBP52 were expressed in HEK cells to assess their interaction with Akt or the Akt-targeting phosphatase PHLPP by co-immunoprecipitation (Fig. 1A and B, suppl. Fig. 1A). While FKBP51 interacted with both Akt and PHLPP<sup>18</sup>, FKBP52 only coprecipitated Akt. The interaction of FKBP51 with both Akt1 and PHLPP required the peptidylprolylisomerase (PPIase) domain (FK1) of FKBP51, but not the enzymatic PPIase activity of this domain ("51PPImut"). These protein interactions did also not require Hsp90 binding of FKBP51 ("Hsp90mut", Fig. 1A and B, suppl. Fig. 1A). To monitor potential functional consequences of these interactions between FKBP51/52 and Akt, we first determined two indicators of Akt activity, i.e. phosphorylation of Akt (S473 on Akt1) and transcriptional activity of FoxO3a. Phosphorylation of Akt at S473 is required for enhanced kinase activity, while phosphorylation at threonine 308 is needed for basal activity. FoxO3a is a forkhead transcription factor which is inhibited by phosphorylation through Akt at serine 318<sup>36</sup>. De-phosphorylation of Akt (S473 on Akt1) was only induced by ectopic expression of FKBP51, but not by FKBP52 (Fig. 1A, suppl. Fig. 1B and C). Phosphorylation of Akt at threonine 308, which is not known as target of PHLPP, remained unchanged under all conditions (suppl. Fig. 1C). The decrease of pFoxO3a paralleled the decrease of pAkt (S473). In addition, only those mutants of FKBP51 that interacted with both Akt and PHLPP also affected phosphorylation of Akt (S473) and FoxO3a (Fig. 1A). These findings support the model that FKBP51, but not FKBP52, recruits PHLPP to Akt through its PPIase domain leading to PHLPP-catalyzed de-phosphorylation of Akt (cf. also Fig. 8).

To assess whether the FKBP51-induced decrease of FoxO3a phosphorylation goes along with altered transcriptional activity of FoxO3a, we performed reporter gene assays in HEK cells.

Transient transfection of either FKBP51 or FKBP52 encoding plasmids in HEK cells revealed a dose-dependent stimulation of FoxO3a-driven reporter gene activity by FKBP51, whereas FKBP52 exerted no or even a mild inhibitory effect (Fig. 1C, suppl. Fig. 1D).

As potential further consequences of FKBP51-triggered inhibition of Akt we analyzed the impact of FKBP51 on autophagic pathways; Akt and the linked mTOR kinase are key regulators of genes involved in autophagy<sup>37, 38</sup>. Comparing FKBP51<sup>-/-</sup> and wild-type (FKBP51<sup>+/+</sup>) mouse embryonic fibroblasts (MEFs), we found that MEFs lacking FKBP51 not only displayed higher levels of Akt phosphorylation (S473), but also higher phosphorylation levels of two downstream targets of mTOR (pp70S6K, p4E-BP1, Fig. 2A and suppl. Fig. 2A). This indicates an overall increased mTOR activity in FKBP51-deficient MEFs. Since mTOR inhibits autophagy pathways, we also determined the expression of three major markers of autophagy. Protein levels of Beclin1, LC3B-II/I and Atg12 were indeed reduced in FKBP51<sup>-/-</sup> MEFs (Fig. 2A). These effects of FKBP51 gene deletion were attenuated by ectopic expression of FKBP51 in FKBP51<sup>-/-</sup> MEFs (Fig. 2A and suppl. Fig. 2A). Together, the data corroborate a stimulatory role of FKBP51 on autophagy pathways.

Given that FKBP51 acts on a variety of intracellular proteins, it was important to test whether the effects of FKBP51 on autophagy pathways require Akt. We used Akt1/2<sup>-/-</sup> MEFs to evaluate FKBP51's ability to affect expression of autophagy markers. In wild-type MEFs, transfection of FKBP51 decreased the phosphorylation levels of the two downstream targets of the Akt-mTOR pathway, pp70S6K and p4E-BP1, and increased the levels of the autophagy markers Beclin1 and LC3B-II/I (Fig. 2B and suppl. Fig. 2B). These effects of FKBP51 were not observed in Akt1/2<sup>-/-</sup> MEFs, but were restored after reintroducing Akt1 and Akt2 into Akt1/2<sup>-/-</sup> by transient transfection (Fig. 2B and suppl. Fig. 2B). We conclude that the impact of FKBP51 on autophagy markers requires Akt1 and/or Akt2 (cf. also Fig. 8).

### *Cellular effects of antidepressants depend on FKBP51*

Since FKBP51 has been linked to the pharmacological responsiveness to antidepressants (ADs) in clinical genetic studies<sup>6, 39</sup>, potential convergent effects of ADs and FKBP51 were analyzed. Both, decrease and increase of pAkt by ADs has been reported to date<sup>12, 13</sup>, and modulation of autophagy pathways has been found for several ADs<sup>14-17</sup>. We used reporter gene assays recording the transcriptional activity of the Akt target FoxO3a in primary rat astrocytes. As observed in HEK cells (Fig. 1C), cotransfection of FKBP51 increased the activity of FoxO3a (Fig. 3A, suppl. Fig. 3A). Treatment with the ADs paroxetine (PAR), amitriptyline (AMI) or fluoxetine (FLX, all at 10  $\mu$ M that proved sufficient for induction of autophagy markers<sup>15</sup>) stimulated reporter gene activity by a factor of  $\sim 1.5$ ; concomitant overexpression of FKBP51 augmented reporter activity by  $\sim 2$ -fold (Fig. 3A, suppl. Figs 3 A and B). Analysis of the phosphorylation status of Akt indicated that FKBP51 and ADs act synergistically to reduce pAkt in primary astrocytes (Fig. 3B, suppl. Figs. 3C and D).

The importance of FKBP51 for AD effects on the Akt-autophagy pathway was further evaluated in FKBP51<sup>-/-</sup> and Akt1/2<sup>-/-</sup> MEFs. The ADs PAR, AMI and FLX reduced phosphorylation of Akt in FKBP51<sup>+/+</sup>, but not in FKBP51<sup>-/-</sup> MEFs; ectopic expression of FKBP51 in FKBP51<sup>-/-</sup> MEFs restored the effect of ADs on Akt phosphorylation (Fig. 3C, suppl. Figs 3E and F). The autophagy marker LC3BII/I was elevated upon AD treatment in FKBP51<sup>+/+</sup> MEF cells (Fig. 3D, suppl. Figs. 3E and G), as reported previously for astrocytes<sup>15</sup>. This AD effect was blunted in FKBP51<sup>-/-</sup> MEFs, and restored when FKBP51 was ectopically expressed in these cells (Fig. 3D, suppl. Figs. 3E and G). In Akt1/2<sup>-/-</sup> MEFs, AD exposure did not induce changes in LC3B-II/I levels (suppl. Figs. 3H and I). When Akt1 and Akt2 were re-introduced by ectopic expression, the effects of ADs re-emerged.

In addition to the conversion of LC3B-I to LC3B-II, the shift of LC3B-II from cytoskeletal to autophagosomal compartments is also a major hallmark of autophagy induction. Therefore, the influence of FKBP51 on the capability of ADs to change the distribution of recombinant

GFP-LC3B was monitored in primary cortical astrocytes. Ectopically expressed FKBP51 enhanced AD-induced clustering of GFP-LC3B (Fig. 3E and F, suppl. Figs. 3J and K). Also in the absence of ADs, FKBP51 triggered the formation of GFP-LC3B positive puncta, although to a lesser extent. FKBP51 also enhanced the ability of ADs to induce conversion from LC3B-I to LC3B-II and to elevate Beclin1 and ATG12 levels in astrocytes (Figs. 3G-I, suppl. Figs. 3C and L-N). Collectively, these data support the conclusion that cellular AD effects on autophagy pathways depend on FKBP51.

#### *Effects of paroxetine in mice depend on FKBP51*

To assess whether FKBP51 also modulates AD response *in vivo*, we treated FKBP51<sup>+/+</sup> and FKBP51<sup>-/-</sup> mice with PAR. Protein extracts from hippocampus and prefrontal cortex showed brain region and genotype dependent responses to PAR. In FKBP51<sup>+/+</sup> mice, PAR treatment (10 mg kg<sup>-1</sup>) evoked de-phosphorylation of Akt, and increased the levels of Beclin1, LC3B-II/I, Atg12 and Vps34. In contrast, FKBP51<sup>-/-</sup> mice revealed no alterations in protein levels of phosphorylated Akt and autophagy markers in either brain region after PAR treatment (Fig. 4A-E, suppl. Figs. 4A and B).

To test whether also the behavioral efficacy of ADs depends on the FKBP51 expression status, we employed a well-established test for AD action; FKBP51<sup>-/-</sup> mice were subjected to a forced swim test to monitor the animals' response to an acute PAR injection. PAR-treated FKBP51<sup>+/+</sup> mice spent less time floating (immobility) compared to vehicle-treated FKBP51<sup>+/+</sup> mice, thus exhibiting the expected response to ADs. In contrast, the effect of PAR was significantly less pronounced in FKBP51<sup>-/-</sup> mice (Fig. 4F right). Similarly, PAR treatment led to an increase in the time spent struggling in FKBP51<sup>+/+</sup> mice compared to vehicle-treated FKBP51<sup>+/+</sup> mice, while this effect of PAR was again significantly attenuated in FKBP51<sup>-/-</sup> mice (Fig. 4F left).

Since autophagy has been linked to neurotransmission, we also tested whether FKBP51 changes the effect of PAR on neuronal function as a physiological correlate of behavior<sup>40</sup>. We electrically stimulated hippocampal CA3-CA1 synapses in brain slices from FKBP51<sup>+/+</sup> and FKBP51<sup>-/-</sup> mice and monitored CA1 neuronal activity by means of voltage-sensitive dye imaging (VSDI)<sup>41-43</sup>. This approach was chosen for two reasons: first, the CA1 area is an important output subfield of the hippocampus, which has been implicated in the pathophysiology of depression and in antidepressant action<sup>44, 45</sup>. Second, we considered potential heterogeneity of the PAR effect at CA3-CA1 synapses; thus, VSDI measurement of neuronal activity in a larger portion of CA1 appeared more promising than the typically more restricted electrode recording techniques. In slices from FKBP51<sup>+/+</sup> mice, bath application of PAR (10  $\mu$ M) enhanced 'region of interest' (ROI)-extracted fast, depolarization-mediated imaging signals (FDSs), which reflect excitatory postsynaptic potentials as well as neuronal action potentials<sup>42, 43</sup>. This effect was not observed in slices from FKBP51<sup>-/-</sup> animals (Fig. 5).

#### *Effects of antidepressants in human blood cells depend on FKBP51*

Finally, we tested whether the link between FKBP51 expression status and AD response of autophagy markers also exists in human peripheral blood mononuclear cells (PBMCs). PBMCs derived from healthy male volunteers showed marked variations in basal FKBP51 expression (Fig. 6). The FKBP51 expression level was negatively correlated with the phosphorylation status of Akt and positively correlated with the autophagy markers Beclin1 and Atg12 (Figs. 6B, C and E); LC3B-II/I displayed a weak correlation with FKBP51 protein levels, while no significant correlation was found for Vps34 (Figs. 6D and F).

In addition, PBMCs derived from healthy male volunteers were also cultivated *ex vivo* and treated with the ADs AMI, FLX and PAR for 72 h. To more closely mimic the clinically relevant *in vivo* conditions, drug concentrations were chosen according to the results of therapeutic drug monitoring in psychiatry<sup>46</sup>. The extent of de-phosphorylation of Akt and

change of LC3B-II/I ratio and levels of Beclin1 in response to ADs correlated with the expression level of FKBP51 (Fig. 7, suppl. Fig. 5). Higher expression of FKBP51 was associated with AD-induced de-phosphorylation of Akt, while low expression of FKBP51 was associated with AD-induced Akt phosphorylation. For Beclin1, a gradual shift from slightly inhibitory to stimulatory effects of ADs was observed with increasing FKBP51 levels. AD-triggered lipidation of LC3B-I was positively correlated with the FKBP51 expression status. Atg12 expression was not significantly changed by AMI and FLX, while PAR affected Atg12 expression in an FKBP51-dependent fashion similarly to Beclin1.

## Discussion

FKBP51 has originally been selected as candidate for genetic analyses in depression and AD responsiveness<sup>6</sup>, because it is involved in the regulation of the glucocorticoid receptor<sup>5</sup>, and thereby the stress hormone axis<sup>47</sup>. Our study brings novel FKBP51-directed pathways to the fore (summarized in Fig. 8) and establishes the importance of FKBP51 in AD action on the molecular, cellular and organismic level. Our data are in line with the observation that the FKBP51 genotype associated with faster AD treatment response is also linked to higher expression levels of FKBP51<sup>6</sup>.

Akt has been reported as target of AD action before, and both positive and negative changes of pAkt levels have been found for different ADs in different cellular systems<sup>12, 13, 48-54</sup>. Based on our *in vitro* and *in vivo* findings, we propose that the FKBP51 expression status determines whether and how antidepressants alter Akt phosphorylation; thus, variations in FKBP51 expression might be the underlying cause for the differences in AD effects on Akt. In general, phosphorylation of Akt is under multifactorial control<sup>55</sup> that could be the basis for both inhibitory and FKBP51-dependent stimulatory actions of antidepressants.

Considering the AD relevant mechanism of action of FKBP51, our protein interaction analyses support the concept of FKBP51 as scaffolding protein that recruits PHLPP to Akt<sup>18</sup>. Mapping of the interaction revealed the FK1 domain as major mediator of the interaction to both Akt and PHLPP. Thus, the impact of FKBP51 on Akt constitutes an Hsp90-independent activity. Hsp90-independent actions of FKBP51 are not without precedence, as intrinsic chaperone activity has been ascribed to FKBP51<sup>56</sup>. Of note, Hsp90 itself is also involved in the regulation of Akt phosphorylation; inhibition of complex formation between Akt and Hsp90 has been shown to promote de-phosphorylation of Akt through PP2A<sup>57</sup> that acts on threonine 308<sup>58</sup>. Our analyses also showed that FKBP52 is interacting with Akt, but not with PHLPP2; accordingly, FKBP52 did not change Akt phosphorylation. Divergent actions of FKBP51 and FKBP52 have also been observed for neurite outgrowth<sup>59</sup>, regulation of



microtubules<sup>60, 61</sup> and control of the corticosteroid receptors<sup>5, 62</sup>. Intriguingly, the FK1 domain has been identified to be crucial for the differential effects on the glucocorticoid receptor<sup>5, 63</sup>, while the enzymatic PPIase activity of FKBP51 was not required for the inhibitory effect<sup>5</sup>. These data collectively raise the possibility that the most relevant function of the FK1 domain of FKBP51, and potentially other PPIase domains, is the establishment of protein-protein interactions, rather than execution of isomerization of peptidylprolyl bonds.

The here established impact of FKBP51 on markers of autophagy significantly expands the range of actions of this versatile protein. Since FKBP51 is regulated by glucocorticoids<sup>64, 65</sup>, this pathway might link physiological stress to autophagy. In fact, it has been reported that the synthetic glucocorticoid dexamethasone induces de-phosphorylation of Akt (serine 473 in Akt1) and autophagy<sup>66, 67</sup>. Impacting autophagy potentially has far reaching cellular and physiological consequences<sup>26</sup>. For example, lower body mass has been reported as one of the consequences of genetically blocking autophagy<sup>68, 69</sup>. Thus, it is possible that the lack of autophagy-promoting actions of FKBP51 in FKBP51<sup>-/-</sup> mice at least partially accounts for the lower body weight of the FKBP51<sup>-/-</sup> versus FKBP51<sup>+/+</sup> animals<sup>4</sup>.

In light of the still unknown molecular mechanism by which ADs exactly act, our results strengthen the view that the achievement of a complete understanding requires the investigation of intracellular AD effects to complement the known actions on mono-aminergic neurotransmitter systems<sup>70</sup>. In general, it is not possible to unequivocally predict complex behavior from distinct cellular events. Nevertheless, a link has been discovered from autophagy to neurotransmission<sup>71</sup>. Thus, it is plausible that FKBP51-governed autophagy pathways underlie also the here described FKBP51 dependency of PAR's effect on neuronal activity in the hippocampus. Furthermore, neuronal circuit dynamics in the hippocampus are linked to behavior in an animal model of depression<sup>45</sup>. This provides supporting evidence that the FKBP51 dependency of PAR's impact on behavior is related to effects on the electrophysiological level and the underlying pathways.

While genetic studies of complex diseases, including studies on respective drug actions, have significantly benefitted from the advanced technologies of genome-wide genotyping, the necessity of complementary approaches such as molecular pathway and network analyses to move from genomic localization to mechanistic insight has come into focus more recently<sup>72, 73</sup>. Our finding of FKBP51-dependent effects of ADs on intracellular pathways strengthens the relevance of the genetic association of FKBP51 with AD response and provides substantial information for more targeted genetic studies. In addition, our results suggest considering autophagy mechanisms as pharmacological target to improve treatment of depression, as discussed for other diseases very recently<sup>31</sup>.

## Figure Legends

**Figure 1.** *Functional interaction of Akt and PHLPP with FKBP51.* **A**, constructs used in the interaction analyses. FK, FK506 binding domain; FL, full length. “PPIaseMut” denotes the F67V/D68V mutation destroying PPIase activity; “Hsp90Mut” denotes the K352A/R356A mutation in the tetratricopeptide repeat (TPR) domain abolishing interaction with Hsp90. HEK cells were transiently transfected with the plasmids encoding the listed FKBP constructs, all fused to a Flag-tag. Precipitation of protein complexes was performed using magnetic protein G beads conjugated to a Flag-antibody and (co)precipitated proteins were visualized by Western blot and quantified (details in Suppl. Fig. 1). Tables summarize the results of 3 independent experiments (3 technical replicates of the Western blots of each experiment) analyzing the interaction of the FKBP5s with Akt1 and PHLPP2 (left) and the change of phosphorylation of Akt (S473 in Akt1) and the Akt target FoxO3a (S318) (right). ++ strong interaction, + weak interaction, - interaction not detectable. ↓↓ strong decrease, ↓ weak decrease, - no change. **B**, representative Western blots of the interaction analyses. Numbers correspond to the constructs listed in A. **C**, The functional effect of FKBP-governed differential FoxO3a phosphorylation was assessed by FoxO3a-driven reporter gene analysis in HEK cells transiently overexpressing increasing amounts of full-length FKBP51 or 52. Expression efficiency was controlled by Western blot analysis (right panel). Relative reporter activity is given in arbitrary units reflecting the mean  $\pm$  SEM of three independent experiments performed in triplicate. \*  $p < 0.05$ ; \*\*\*  $p < 0.001$ , statistical details in suppl. Fig. 1.

**Figure 2.** *FKBP51 dependency of Akt pathways.* **A**, The impact of the FKBP51 expression status on the levels of pp70S6K, p4E-BP1, pAKT1 (S473), Beclin1, LC3B-II/I, and Atg12 was assessed by Western blot analysis in FKBP51<sup>+/+</sup> MEFs, FKBP51<sup>-/-</sup> MEFs, and FKBP51<sup>-/-</sup> MEFs transfected with an FKBP51 expressing plasmid. Graphs show the relative mean expression  $\pm$  SEM of 4 different experiments; expression in FKBP51<sup>+/+</sup> MEFs was set to 1. **B**,

the dependency of FKBP51's impact on the expression of pp70S6K, p4W-BP1, Beclin1, and LC3B-II/I was evaluated in Akt1/2<sup>+/+</sup> MEFs, Akt1/2<sup>-/-</sup> MEFs and in Akt1/2<sup>-/-</sup> MEFs transfected with Akt1 and Akt2 expressing plasmids. Graphs display pairwise comparisons of cells cotransfected with vector or FKBP51 expressing plasmid and represent the relative mean expression + SEM of 3 different experiments; expression in Akt1/2<sup>+/+</sup>, vector-transfected MEFs was set to 1. \* p<0.05; \*\* p<0.01; \*\*\* p<0.001. See suppl. Table 1 for all statistical parameters.

**Figure 3.** *FKBP51 enhances antidepressants' effects in cells.* **A**, primary rat cortical astrocytes were transfected with a FoxO3a-driven reporter plasmid in combination with an FKBP51 expressing vector or control vector. Treatment with PAR (10  $\mu$ M) was for 48 h. Bars represent the mean + SEM of 3 independent experiments performed in triplicate; reporter activity in untreated, control-transfected cells was set to 1. **B**, primary rat astrocytes were transfected with an FKBP51 expressing vector or control vector, treated with PAR (10  $\mu$ M) for 48 h, and the pAkt/Akt ratio was determined by Western blot. Bars represent the mean + SEM of 3 independent experiments performed in triplicate. pAkt/Akt in untreated, control-transfected cells was set to 1. **C**, **D**, FKBP51<sup>+/+</sup>, FKBP51<sup>-/-</sup> and FKBP51<sup>-/-</sup> MEFs transfected with an FKBP51 expressing plasmid were treated with PAR (10  $\mu$ M) for 48 h, and the pAkt/Akt (**C**) and LC3B-II/I (**D**) ratios were determined by Western blot. Bars represent the mean + SEM of 3 independent experiments performed in triplicate. Protein levels in FKBP51<sup>+/+</sup>, untreated cells were set to 1. **E**, primary rat cortical astrocytes were transfected with a vector expressing GFP-LC3B, in combination with an FKBP51 expressing vector or cloning vector, and treated with PAR (10  $\mu$ M) for 48 h. The number of GFP-LC3B positive puncta was determined per cell. 15-25 randomly selected cells were evaluated for each condition. **F**, representative fluorescence images of **E**. **G-I**, cortical astrocytes were transfected and treated as in **B** and LC3B-II/I Beclin1 levels were determined by Western

blotting. Bars represent the mean + SEM of 3 independent experiments performed in triplicate. Protein levels in wild-type, untreated cells were set to 1. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . See suppl. Table 1 for all statistical parameters.

**Figure 4.** *FKBP51 determines the effect of PAR in mice.* **A-E**, FKBP51<sup>-/-</sup> and FKBP51<sup>+/+</sup> mice were treated with PAR (10 mg kg<sup>-1</sup>) or vehicle and sacrificed 45 mins later. Levels of the indicated proteins were determined by Western blotting in extracts of the hippocampus and the prefrontal cortex from 9-10 animals in technical triplicates. **F**, FKBP51<sup>-/-</sup> and FKBP51<sup>+/+</sup> mice were treated with PAR or vehicle and subjected to the forced swim test (N = 7-9 per group). Graphs show the times of immobility and struggling. See suppl. Table 1 for all statistical parameters. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . See suppl. Table 1 for all statistical parameters.

**Figure 5.** *The effect of PAR on evoked CA1 neuronal activity depends on FKBP51.* **A**, Left, illustration of the position of the stimulation electrode (Stim) and ROI used for the calculation of CA1 neuronal population activity. Right, depolarization-mediated VSDI signal recorded 11 ms after the electrical stimulation pulse. Warmer colors represent higher values of the fractional change in fluorescence ( $\Delta F/F$ ) and, thus, stronger neuronal activity. **B**, Left, quantification of PAR's effects on CA1 FDSs (N= 10 slices from 4 mice for each group). \*  $p = 0.032$  (paired  $t$ -test); n.s.,  $p = 0.912$  (paired  $t$ -test); data are given as mean  $\pm$  SEM. Right, representative recording traces illustrating the PAR effects on CA1 FDSs.

**Figure 6.** *Expression of FKBP51 in human peripheral blood lymphocytes varies and correlates with pAkt-autophagy pathway components.* Peripheral blood mononuclear lymphocytes were collected from healthy male volunteers (N=20) and protein levels were determined in cell extracts by Western blotting (example blot shown in A). Plots display the

correlation between the levels of FKBP51 and pAkt (S473), Beclin1, LC3B-II/I, Atg12 or Vps34. Each dot represents the levels of FKBP51 and the respective protein in PBMCs from one individual. Average expression levels were set to 1.

**Figure 7.** *The effects of antidepressants in human peripheral blood lymphocytes correlate with the expression level of FKBP51.* Peripheral blood mononuclear cells were collected from healthy male volunteers (N=20), cultivated *ex vivo* and treated with AMI (0.888  $\mu$ M), FLX (1.695  $\mu$ M) or PAR (0.365  $\mu$ M) for 48 h. The levels of FKBP51, pAkt (S473), Beclin1, LC3B-II/I, and Atg12 were determined by Western blots. Plots depict the change in protein levels upon treatment with AD compared to vehicle-treated cells in correlation to the expression level of FKBP51. Each dot represents the levels of FKBP51 and the change of the levels of the respective protein in PBMCs from one individual determined in treated and untreated cells.

**Figure 8.** *Model of FKBP51's impact on Akt, mTOR and autophagy pathways.* **A**, through its PPIase domain (FK1), FKBP51, but not FKBP52, recruits PHLPP to Akt, thereby inactivating Akt through de-phosphorylation (S473 in Akt1). Inhibition of Akt removes its inhibitory action on FoxO3a (**B**) and abolishes the stimulatory effect on mTOR (**C**). The resulting loss of mTOR activity leads to reduced phosphorylation of p70S6K (**D**) or 4E-BP1 (**E**), respectively; it also removes the suppressive effect of mTOR on autophagy (**F**), indicated by the increase of several markers. Antidepressants act on the same pathways in an FKBP51-dependent manner.

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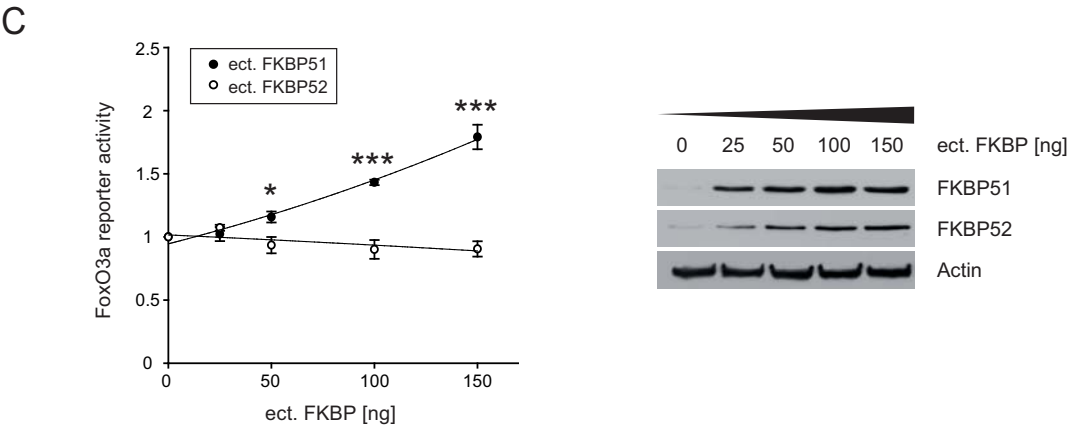
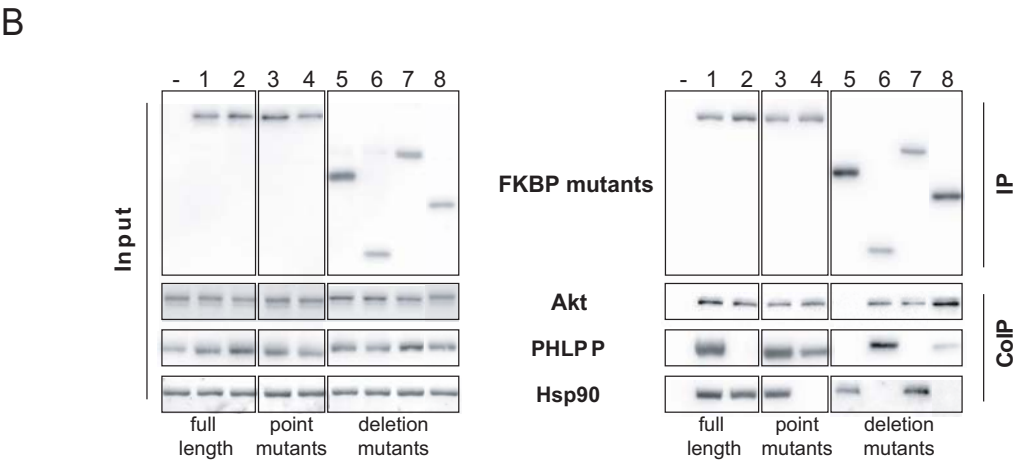
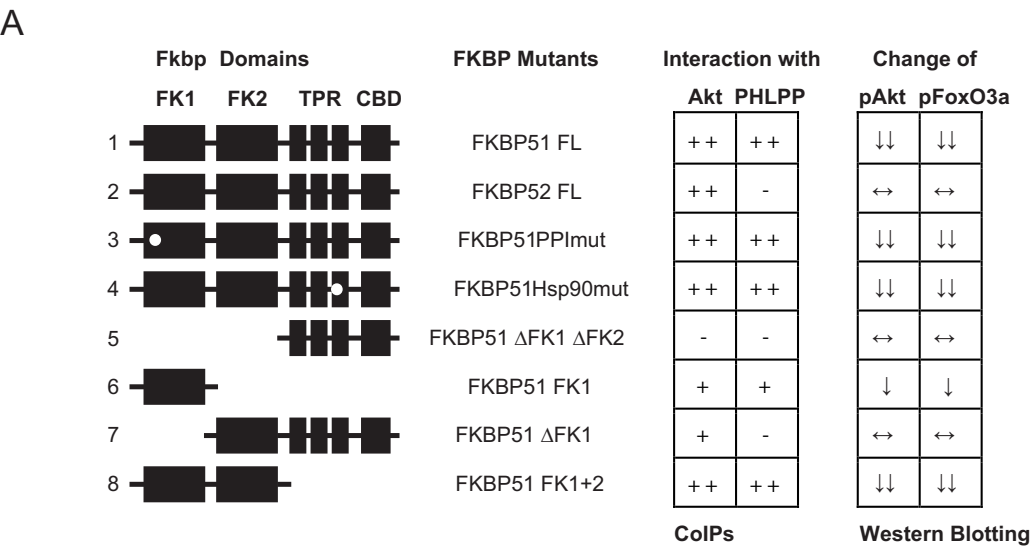


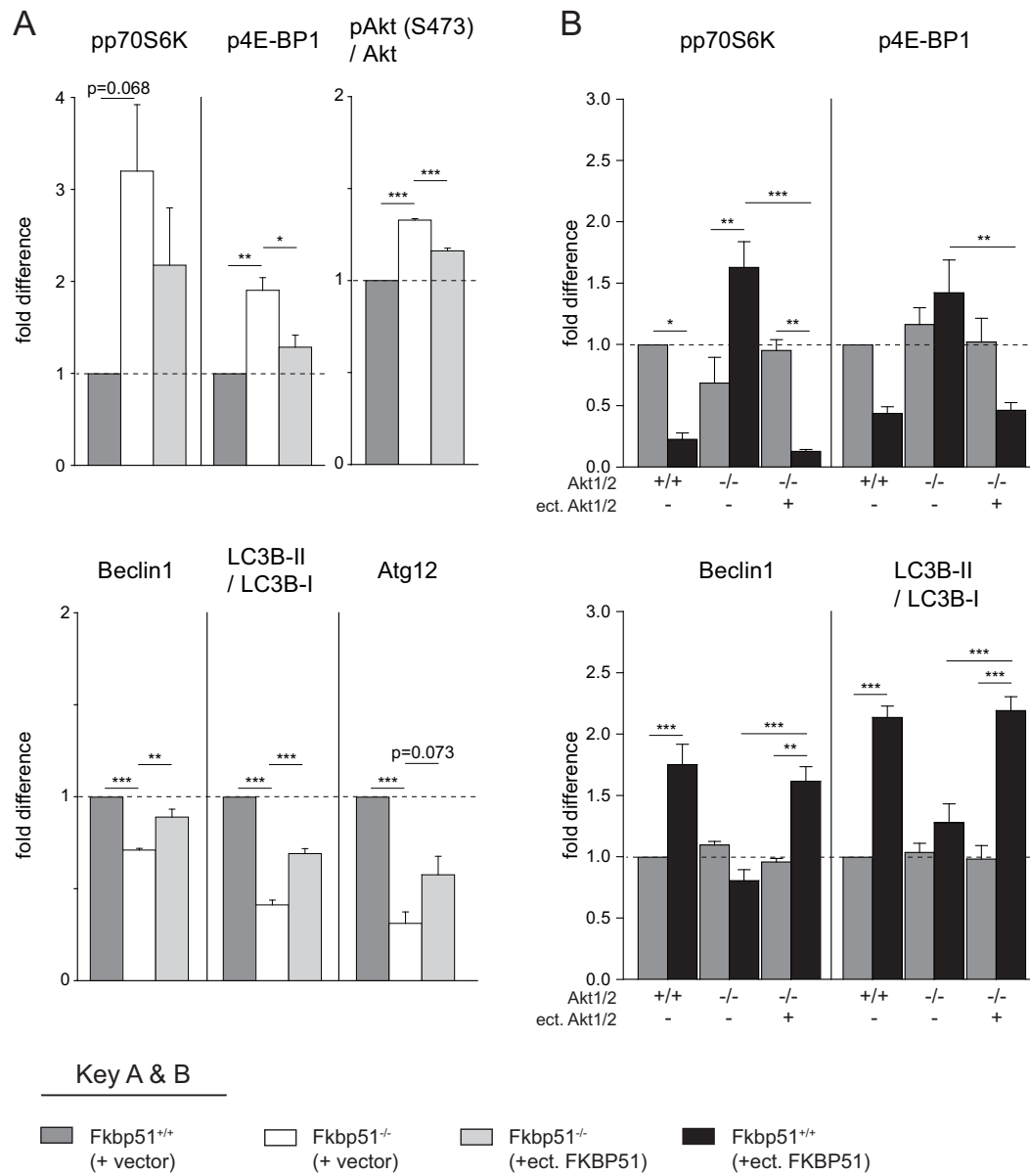
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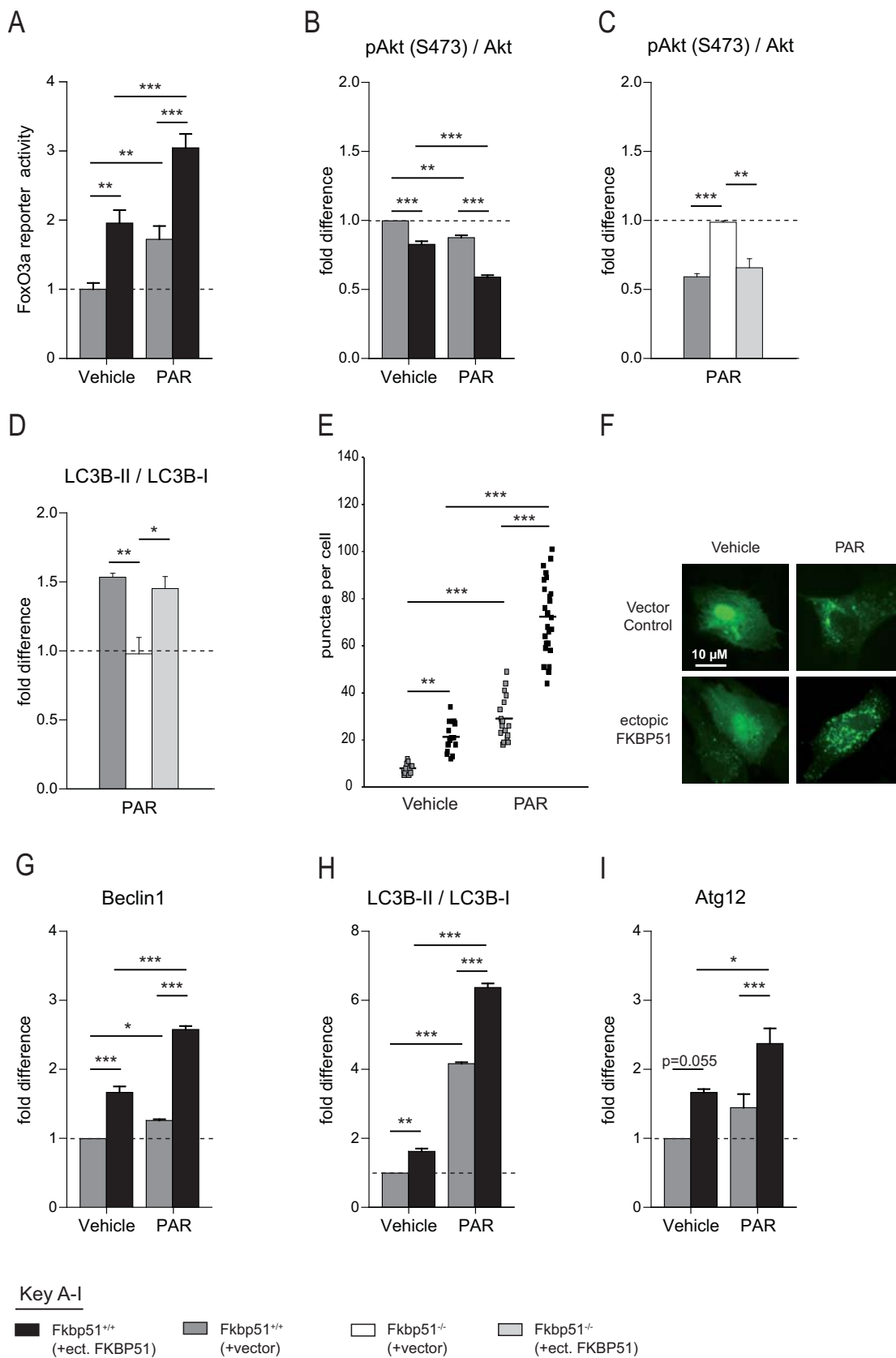
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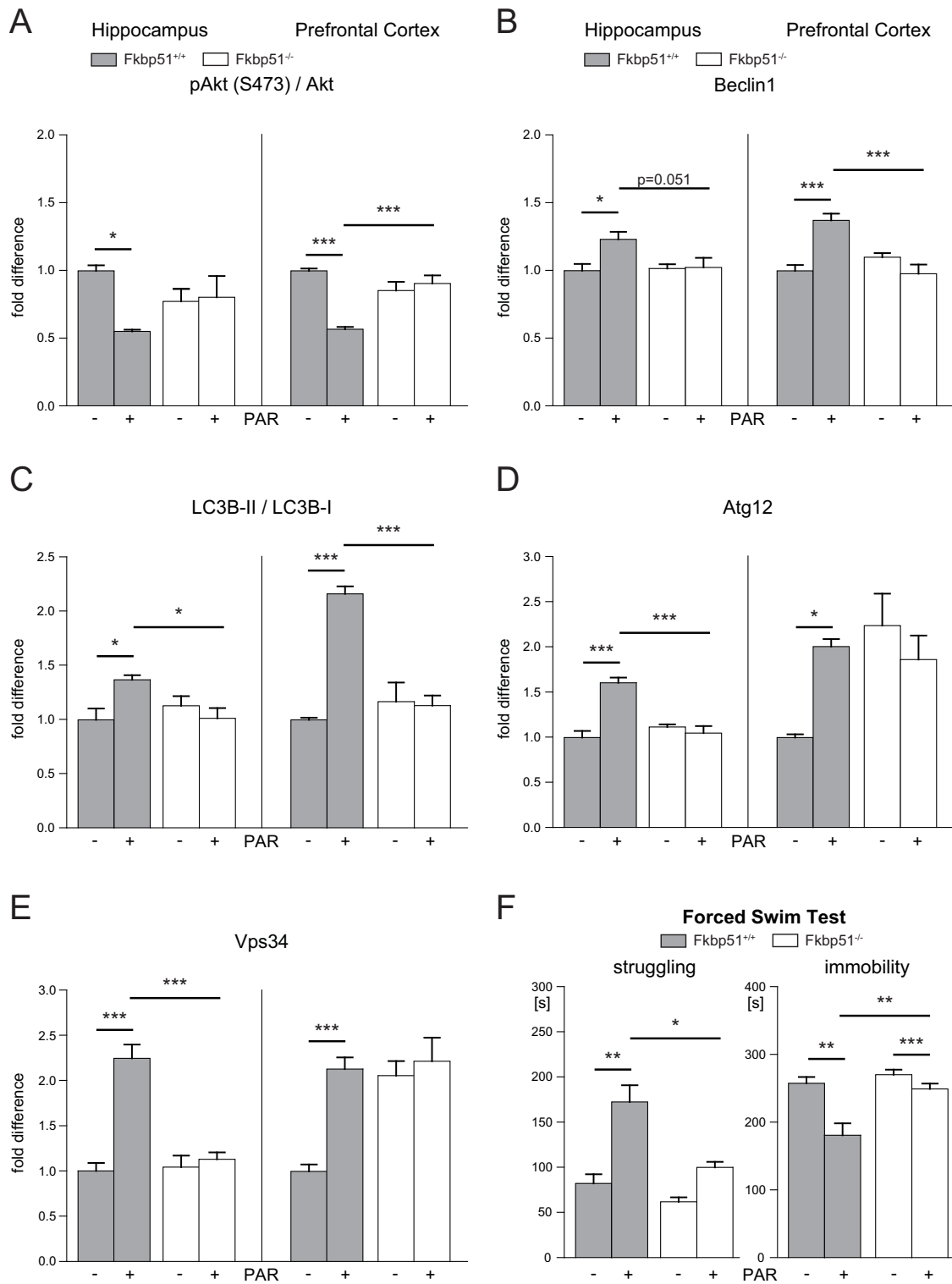
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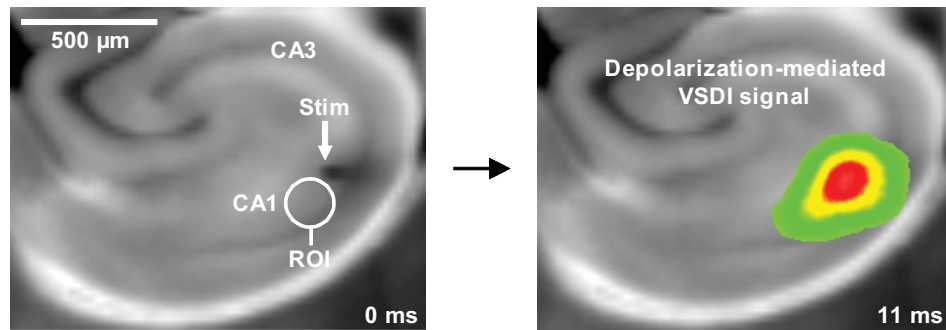




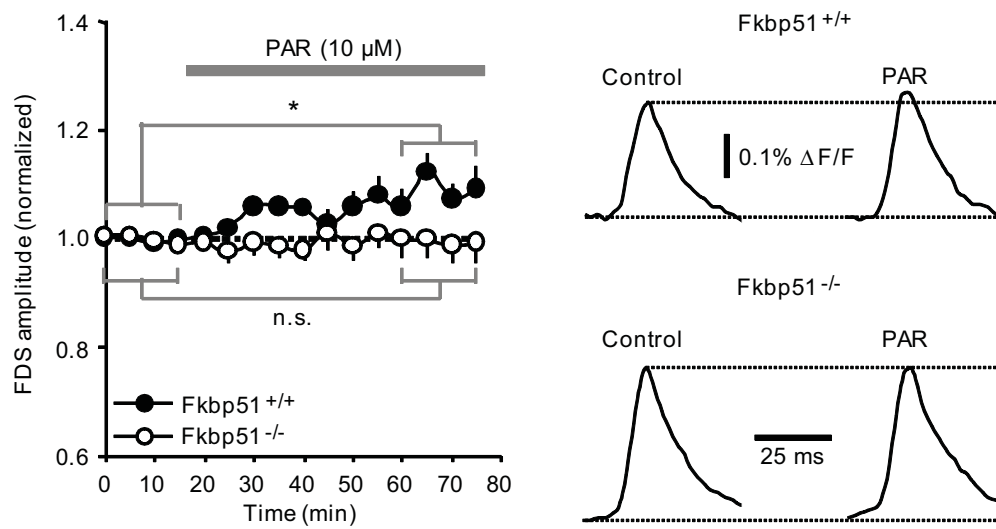




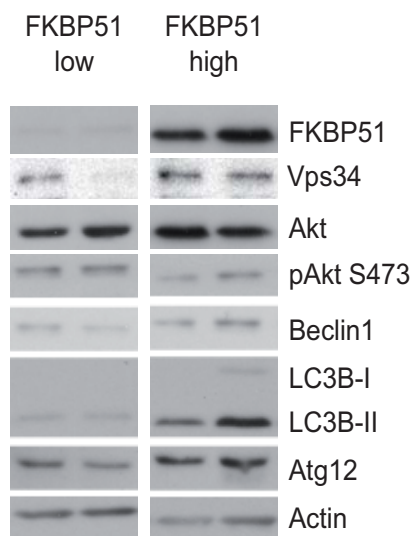
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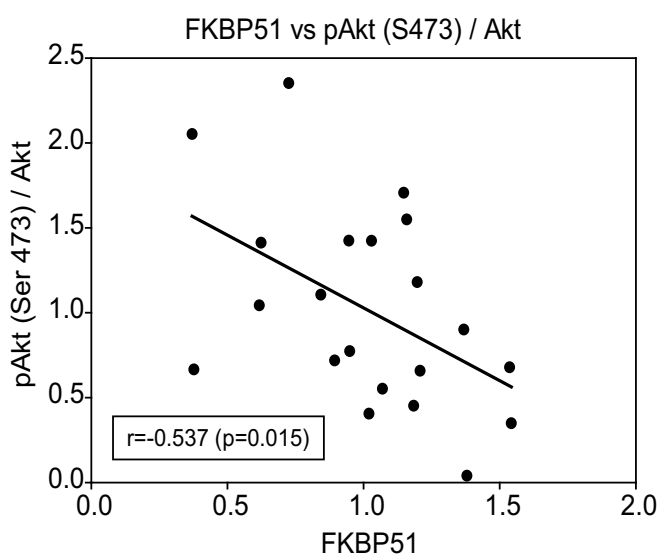
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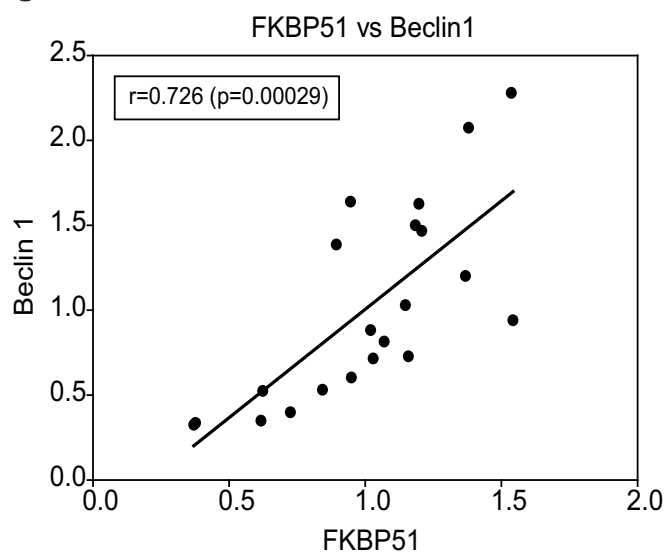
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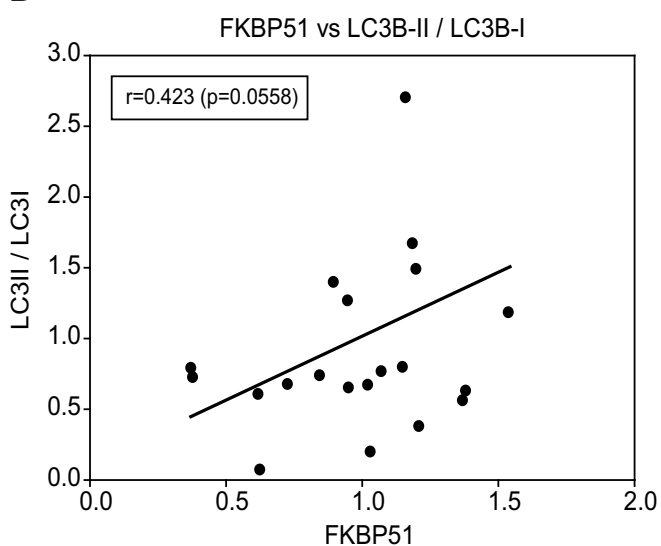
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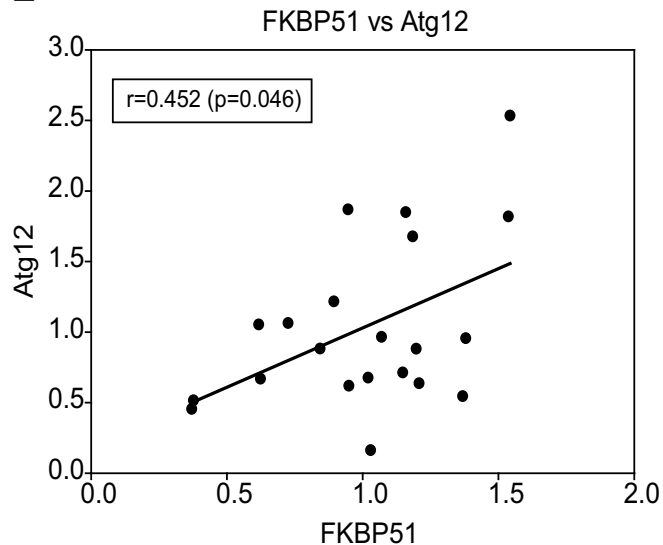
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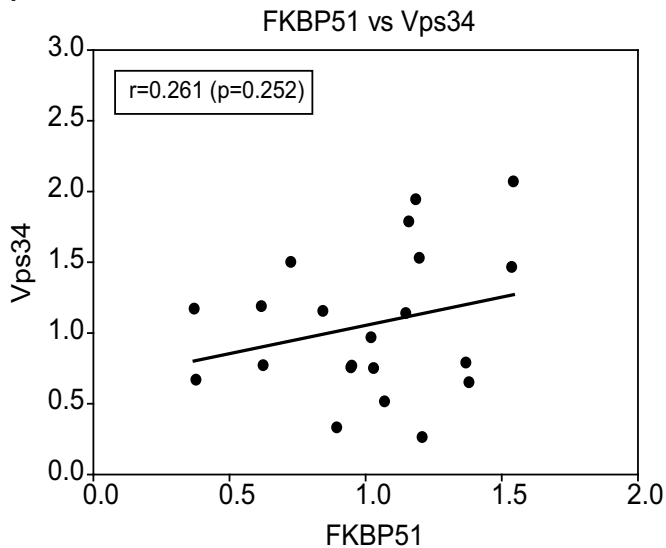
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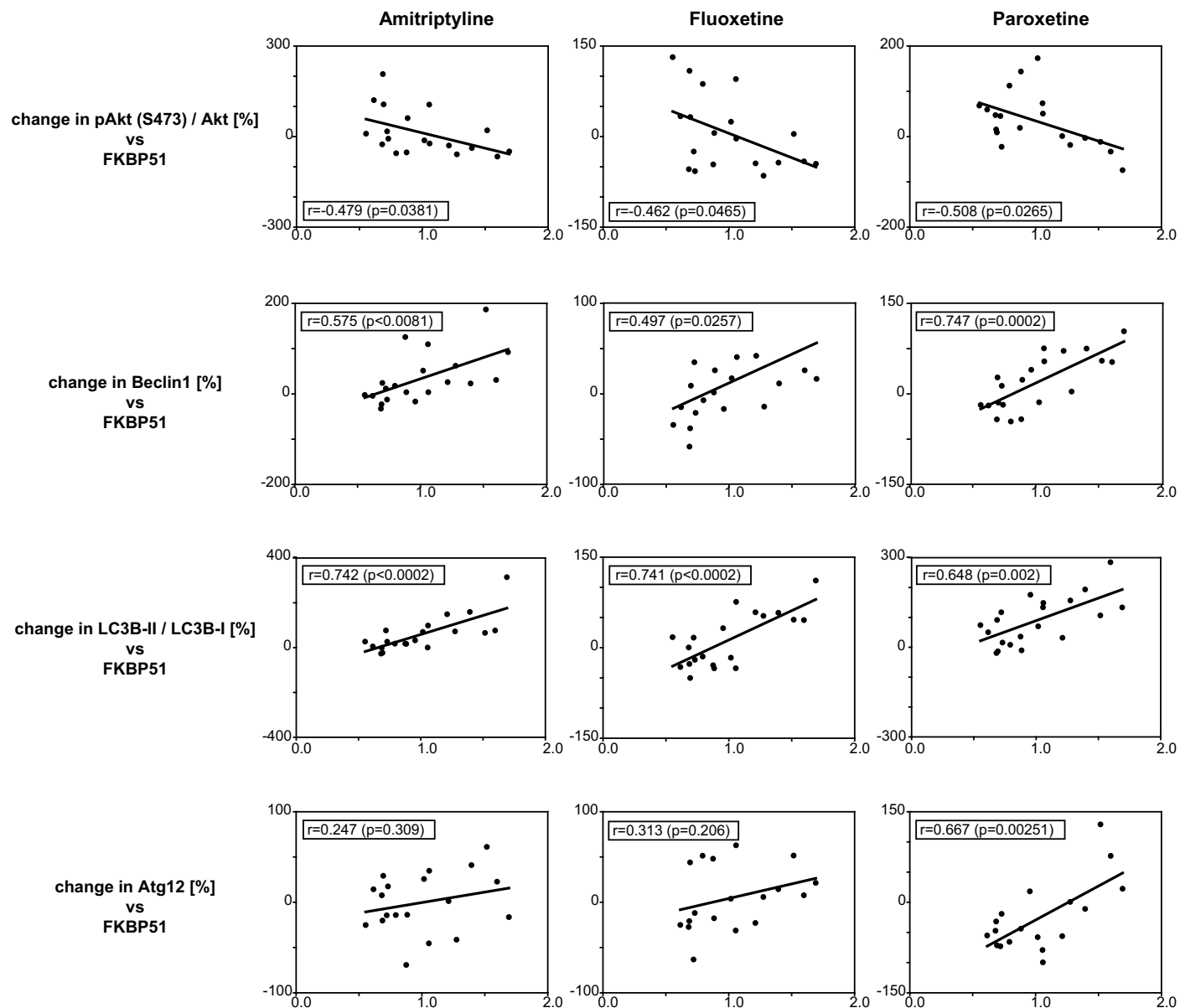


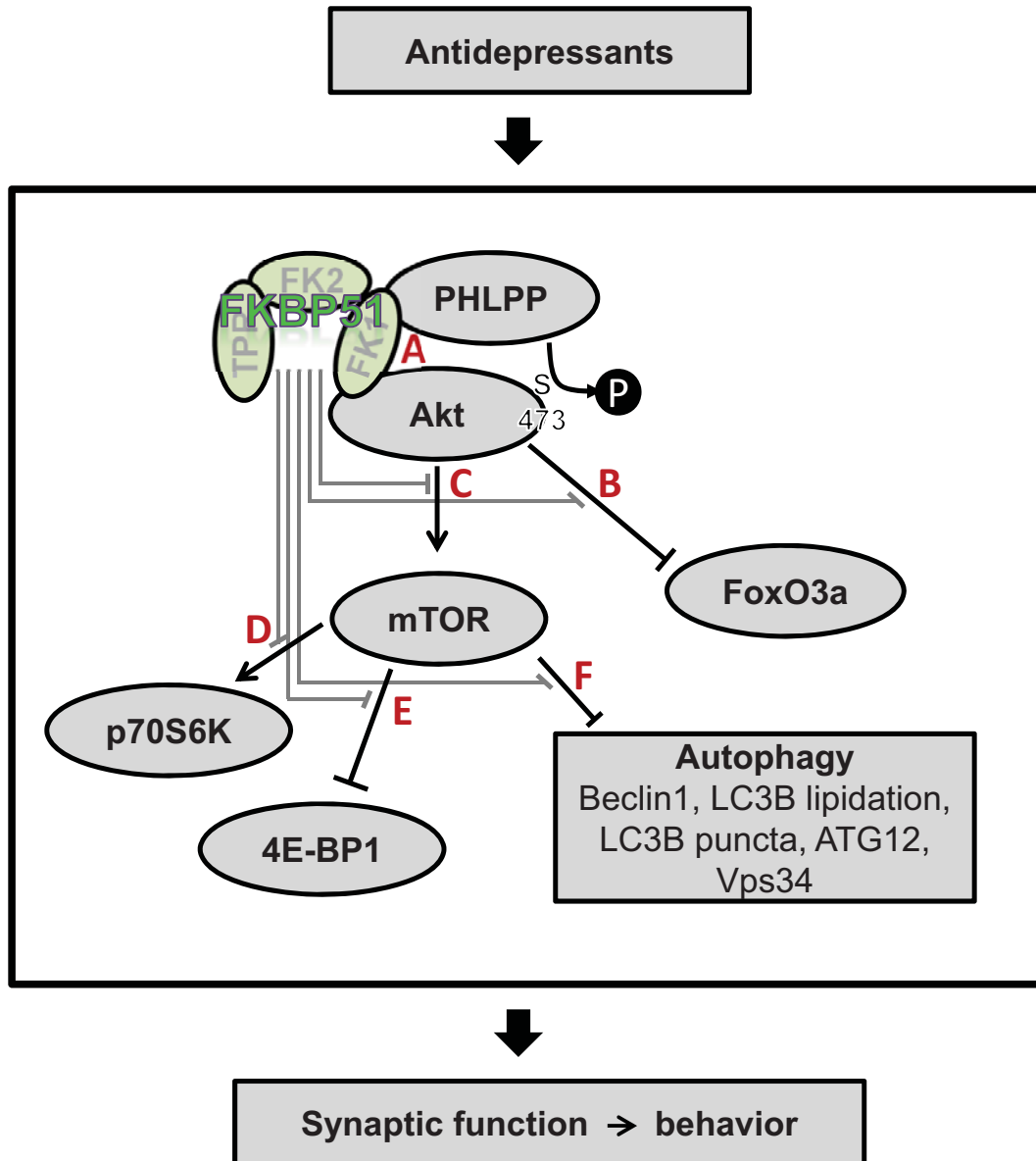
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**F**







## Supplemental Material to Gassen et al., FKBP51 shapes antidepressant actions

### LEGENDS TO SUPPLEMENTAL FIGURES

**Suppl. Figure 1.** *Summary of data of the functional interaction of Akt and PHLPP with FKBP51.* **A**, HEK cells were transiently transfected with the plasmids encoding the FKBP constructs listed in figure 1 and analyses of interaction with Akt, PHLPP, and Hsp90 were performed by co-immunoprecipitation and Western blot analysis. Interaction with wild-type full-length FKBP51 was set to 1 for all interactions and served as reference for interaction with the other FKBP isoforms. The table summarizes the results of 3 independent co-immunoprecipitations with 3 technical replicates of the respective Western blots. Numbers indicate relative intensity of interacting proteins, including p-values of interaction differences (compared to full-length wt FKBP51). **B**, representative Western blots for Figure 1A, change of pAkt and pFoxO3a. The FKBP proteins were detected by an antibody directed against their FLAG tags. **C**, summary of the numeric results of the effect of FKBP51 expression in HEK cells on the phosphorylation status of Akt1 and FoxO3a (corresponding to Fig. 1A). Numbers reflect the results of 3 independent experiments with 3 technical replicates of the respective Western blots. Numbers indicate the phosphorylation level of Akt1 or FoxO3a (normalized to the Western signal of the respective total protein), with the level of vector-transfected cells (mutant “-”) set to 1, including p-values of phosphorylation changes. p-values < 0.05 are underlined in A and C. **D**, List of p-values corresponding to Fig. 1C.

**Suppl. Figure 2** *Cellular effects of FKBP51 require Akt1 or Akt2.* Representative Western blots documenting the impact of FKBP51 on pp70S6K, p4W-BP1, pAKT (S473), Beclin1,

LC3B-II/I, and Atg12. Examples correspond to figures 2A and B of the main text. See suppl. Table 1 for all statistical parameters.

**Suppl. Figure 3.** *Convergent effects of FKBP51 and antidepressants.* **A and B**, primary rat cortical astrocytes were transfected with a FoxO3a-driven reporter plasmid in combination with an FKBP51 expressing vector or control vector and treated with AMI, FLX or PAR (10  $\mu$ M each) for 48 h. Representative Western is shown (A). Bars represent the mean + SEM of 3 independent experiments performed in triplicate (B); reporter activity in untreated, control-transfected cells was set to 1. **C, D, L-N**, cortical astrocytes were transfected and treated as in A,B and levels of pAkt/Akt (D), Beclin1 (L), LC3B-II/I (M) and Atg12 (N) were determined by Western blotting (representative blot in C). Bars represent the mean + SEM of 3 independent experiments performed in triplicate. Protein levels in wild-type, untreated cells were set to 1. **E-G**, FKBP51<sup>+/+</sup>, FKBP51<sup>-/-</sup> and FKBP51<sup>-/-</sup> MEFs transfected with an FKBP51 expressing plasmid were treated AMI, FLX or PAR (10  $\mu$ M each) for 48 h, and the pAkt1(S473)/Akt1 (F) and LC3B-II/I (G) ratios were determined by Western blot (representative blot shown in E). Bars represent the mean + SEM of 3 independent experiments performed in triplicate. Protein levels in FKBP51<sup>+/+</sup>, untreated cells were set to 1. **H-I**, Akt1/2<sup>+/+</sup> MEFs, Akt1/2<sup>-/-</sup> MEFs and in Akt1/2<sup>-/-</sup> MEFs transfected with Akt1 and Akt2 expressing plasmids were treated with AMI, FLX or PAR (10  $\mu$ M each) for 48 h. The ratio of LC3B-II/I was determined by Western blot (representative example in H). Bars represent the mean + SEM of 3 independent experiments performed in triplicate. LC3B-II/I in the respective untreated cells was set to 1 (dotted line). **J and K**, primary rat cortical astrocytes were transfected with a vector expressing GFP-LC3B, in combination with an FKBP51 expressing vector or cloning vector, and treated with AMI or FLX (10  $\mu$ M each) for 48 h. The number of GFP-LC3B positive puncta was determined per cell. 15-25 randomly selected

cells were evaluated for each condition. **K**, representative fluorescence images of J. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . See suppl. Table 1 for all statistical parameters.

**Suppl. Figure 4.** *The effect of paroxetine in mice depends on FKBP51.* FKBP51<sup>-/-</sup> and wild-type mice were treated with PAR or vehicle and sacrificed 45 mins later. **A**, **B**, representative Western blots to figure 4 of the main text.

**Suppl. Figure 5.** Representative Western of extracts from antidepressant-treated peripheral blood mononuclear lymphocytes (corresponding to figure 7 of the main text).

**Suppl. Table.** Details of results of statistical analyses. NS, non significant.

## **MATERIALS AND METHODS**

**Cell lines** - Human embryonic kidney cells (HEK-293, ATCC CRL-1573) and mouse embryonic fibroblasts (MEFs) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FCS and 100 units/ml penicillin and streptomycin, respectively. FKBP51<sup>-/-</sup> and Akt1/2<sup>-/-</sup> MEFs (kind gift of Nissim Hay, University of Illinois, Chicago, Illinois) have been described before<sup>1, 2</sup>.

**Primary cultures of murine astrocytes** - Enriched astroglial cultures were prepared from postnatal day 1 rat pups (Sprague-Dawley, Charles River, Sulzfeld, Germany) and handled as described<sup>3, 4</sup>.

**FKBP51 Rescue in MEF Fkbp51<sup>-/-</sup> cells** – Afore detached MEF cells ( $2 \times 10^6$ ) were re-suspended in 100  $\mu$ l transfection buffer (50 mM HEPES pH 7.3, 90 mM NaCl, 5 mM KCl, 0.15 mM CaCl<sub>2</sub>)<sup>5</sup>. A maximal amount of 5  $\mu$ g plasmid DNA was added to the cell suspension, and electroporation was carried out using the Amaxa Nucleofactor system (program # T-020). Cells were re-plated at a density of  $10^5/\text{cm}^2$  and further processed for Western blot analysis.

**Analysis of GFP-LC3 in astrocytes** -  $2 \times 10^6$  cells were transfected with 2 ng GFP-LC3 expressing plasmid or the respective cloning vector using Amaxa Nucleofactor system as described above. Cells were grown for 48h and analyzed by fluorescence microscopy. At least 20 cells were counted.

**Preparation of human PBMCs** – Blood of healthy male volunteers was collected via venipuncture, diluted with PBS and carefully loaded on Biocoll solution (1BioChrom AG, L6113) and centrifuged at  $800 \times g$  for 20 min (brakeless running down). PBMCs were enriched by selecting the interphase of the Biocoll gradient. PBMCs of the interphase were washed two times with ice-cold PBS. PBMCs were re-suspended in RPMI and plated at  $4 \times 10^5/\text{cm}^2$ . After recovery for 6 h, cells were treated with either 888 nM (120 ng ml<sup>-1</sup>) AMI, 1695 nM (500 ng ml<sup>-1</sup>) FLX, 365 nM (120 ng ml<sup>-1</sup>) PAR. Concentrations had been chosen to match therapeutic concentrations in the serum according to the consensus guidelines for therapeutic drug monitoring in psychiatry<sup>6</sup>.



**Plasmids** – FKBP51 deletion mutants were constructed using the pRK5-FKBP51 plasmid as template<sup>7</sup>. PCR was performed as described before<sup>7</sup> with the following combination of primers; dFK1, fwd5'-TCTCTCCACTCGAGATA TGAAAGGAGAGGATTTATTTG-3, rev 5'-CTCTTCTTGCGGCCGCTCACTTGTCATCGTCGTCCTTGTAGTCTACGTGGCCC TCAGGTTTCTC-3'; dFK1+FK2, fwd5'-TCTCTCCA CTCGAGATA TGAAGGCCAAAGAATCCTGGG-3', rev 5'-CTCTTCTTGCGGCCGCTCACTTGTCATCGTCGTCCTTGTAGTCTACGTGGCCC TCAGGTTTCTC-3'; FK1+FK2, fwd5'-TTCTTTCCA CTCGAGATA TGA CTA CTG ATG AAG G-3', rev. 5'-CTCTTCTTGCGGCCGCTCACTTGTCATCGTCGTCCTTGTAGTCTTTCGAAGCTCTTAAGTGTAAC-3'; FK2, fwd5'-TCTCTCCACTCGAGATA TGAAAGGAGAGGATTTATTTG-3, rev 5'-CTCTTCTTGCGGCCGCTCACTTGTCATCGTCGTCCTTGTAGTCTTTCGAAGCTCTTAAGTGTAAC-3'. All clones were checked by Sanger sequencing.

Plasmids expressing Akt1, Akt2, LC3B, FoxO3a-Luc, and PHLPP2-HA have been described<sup>8-10</sup>.

**Reporter gene assays** - Reporter gene experiments were carried out in 96-well formats.  $2 \times 10^6$  cells were transfected with 1000 ng FoxO3a-Luc, 100 ng pCMV-Gaussia-Luc and 1000 ng pRK5-FKBP51/52-Flag -plasmid using Amaxa Nucleofactor system as described above. To determine Firefly luciferase activity, cells were lysed in 50  $\mu$ L passive lysis buffer (0.1 M KPO<sub>4</sub> pH 7.8, 0.2% Triton); addition of 50  $\mu$ L of luciferase reaction buffer to a fraction of 10  $\mu$ L of cell lysate (33 mM KHPO<sub>4</sub>, pH 7.8, 1.7 mM ATP, 3.3 mM MgCl<sub>2</sub>, and 13 mM luciferin) and luminometric readings were performed with an automatic counter (Tristar, Berthold). Activity of secretory Gaussia luciferase<sup>11</sup> was measured after addition of 50  $\mu$ L of substrate/buffer (1.1 M NaCl, 2.2 mM EDTA, 0.22 M KPO<sub>4</sub> pH 5.1, 0.44 mg ml<sup>-1</sup> BSA, and 0.5 mg ml<sup>-1</sup> coelenterazine).

**Co-Immunoprecipitation(CoIP)** - CoIP of FLAG-tagged FKBP51 mutants with endogenous Akt1 with FLAG-tagged FKBP51/52 were performed in HEK-293 cells.  $5 \times 10^6$  cells were electroporated with 5  $\mu$ g of the respective expression plasmids using a GenePulser (Bio-Rad, USA) at 350 V/700  $\mu$ F in 400  $\mu$ l of electroporation buffer (50 mM  $K_2HPO_4/KH_2PO_4$ , 20 mM KAc, pH 7.35, 25 mM  $MgSO_4$ ). After three days of cultivation in DMEM/10% FCS, cells were lysed in CoIP-buffer containing 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Igepal complemented with protease inhibitor cocktail (Sigma, P2714). This was followed by incubation on an overhead shaker for 20 min at 4 °C. The lysate was cleared by centrifugation, the protein concentration was determined, and 1.2 mg of lysate was incubated with 2.5  $\mu$ g FLAG antibody overnight at 4 °C. 20  $\mu$ l of BSA-blocked Protein G Dynabeads (Invitrogen, 100-03D) were added to the lysate–antibody mix followed by 3 h incubation at 4 °C. The beads were washed 3 times with PBS and protein-antibody complexes were eluted with 100  $\mu$ l of 1 $\times$  FLAG-peptide solution (Sigma, 100–200  $\mu$ g ml<sup>-1</sup>, F3290) in CoIP buffer for 30 min at 4 °C. 5–15  $\mu$ g of the cell lysates or 2.5  $\mu$ l of the immunoprecipitates were separated by SDS-PAGE.

**Western Blot analysis** – Protein extracts were obtained by lysing cells in 62.5 mM Tris, 2% SDS and 10% sucrose, supplemented with protease (Sigma , P2714) and phosphatase (Roche, 04906837001) inhibitor cocktail. Samples were sonicated and heated at 95 °C for 5 min. Proteins were separated by SDS-PAGE and electro-transferred onto nitrocellulose membranes. Blots were placed in Tris-buffered saline, supplemented with 0.05% Tween (Sigma, P2287) and 5% non-fat milk for 1 h at room temperature and then incubated with primary antibody (diluted in TBS/0.05% Tween) overnight at 4 °C. The following primary antibodies were used: Beclin1 (1:1000, Cell Signaling, #3495), Atg12 (1:1000, Cell Signaling #2010), LC3B-I/II (1:1000, Cell Signaling, #2775), FLAG (1:7000, Rockland, 600-401-383), HA-HRP conjugated (1:25000, Roche, 11667475001), FKBP51 (1:1000, Bethyl, A301-430A ), FKBP52 (1:2000, Bethyl, A301-427A), Akt (1:1000, Cell Signaling, #4691), pAkt (1:1000,

Ser472, T308, 1:1000, Cell Signaling, #4058, #9275), pFoxO3a (1:1000, Cell Signaling, #9465), PI3K Class III (Vps34, 1:1000, Cell Signaling, #4263), p4E-BP1 (Thr37/46, 1:1000, Cell Signaling, #2855), pp70S6K (Thr371, 1:1000, Cell Signaling, #9208), Actin (1:5000, Santa Cruz Biotechnologies, sc-1616).

Subsequently, blots were washed and probed with the respective horseradish peroxidase- or fluorophore-conjugated secondary antibody for 1 h at room temperature. The immunoreactive bands were visualized either using ECL detection reagent (Millipore, Billerica, MA, USA, WBKL0500) or directly by excitation of the respective fluorophore. Determination of the band intensities were performed with BioRad, ChemiDoc MP, or X-ray-films

**Animals and animal housing** – The Fkbp51 knockout (Fkbp51<sup>-/-</sup>) mouse line was previously generated<sup>1, 12</sup> and fully backcrossed to C57/Bl6. Genotypes were verified by PCR of tail DNA. Only male mice were used for the experiment, obtained from heterozygous breeding pairs. Animals were between 10 and 16 weeks old at the start of the experiment. Mice were held under standard conditions (12L: 12D light cycle, lights on at 08:00 am, temperature 23±2°C), were singly housed and acclimated to the room for one week before the beginning of the experiments. Food (Altromin 1314, Altromin GmbH, Germany) and tap water were available *ad libitum*.

All experiments were carried out in the animal facilities of the Max Planck Institute of Psychiatry in Munich, Germany. The experiments were carried out in accordance with the European Communities' Council Directive 86/609/EEC. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory animals of the Government of Upper Bavaria, Germany.

**Animal treatments and behavioral experiments** – All mice were injected intraperitoneally either with a single dose of paroxetine (10 mg kg<sup>-1</sup>; dissolved in saline; Sigma-Aldrich, Germany) or saline vehicle solution. The injection volume was 5 ml kg<sup>-1</sup> body weight. 45 min after the injection, a subgroup of mice (WT vehicle, n = 10; Fkbp51<sup>-/-</sup> vehicle, n = 9; WT paroxetine, n = 10; Fkbp51<sup>-/-</sup> paroxetine, n = 10; WT) was sacrificed by decapitation following quick anesthesia by isoflurane. Trunk blood was collected in 1.5 ml EDTA-coated microcentrifuge tubes (KabeLabortechnik, Germany) and kept on ice until further processing. Furthermore, brains were removed; hippocampus and prefrontal cortex were extracted and stored on dry ice until further processing.

Another subgroup of mice (WT vehicle, n = 8; Fkbp51<sup>-/-</sup> vehicle, n = 8; WT paroxetine, n = 9; Fkbp51<sup>-/-</sup> paroxetine, n = 7) was subjected to a forced swim test (FST) 45 min after the injection. The FST was carried out between 9:00 am and 10:30 am in the same room in which the mice were housed and was analyzed using an automated video-tracking system (Anymaze 4.20, Stoelting, IL; USA). In the FST, each mouse was put into a 2 l glass beaker (diameter: 13 cm, height: 24 cm) filled with tap water (21 ± 1 °C) to a height of 15 cm, so that the mouse could not touch the bottom with its hind paws or tail. Testing duration was 6 min. Time spent immobile and time spent struggling was scored by an experienced observer, blind to genotype or treatment of the animals.

**Electrophysiology** - Brain slices were obtained from 11-18 week-old male FKBP51<sup>+/+</sup> and FKBP51<sup>-/-</sup> mice. Preparation and staining of slices with the voltage-sensitive dye Di-4-ANEPPS as well as VSDI and data analysis were performed as described<sup>13, 14</sup>, with the exception that  $\Delta F/F$  values were smoothed using a 5 x 5 x 3 average filter. To reduce noise, four acquisitions subsequently recorded at intervals of 5 s were averaged. Neuronal activity was evoked by square pulse electrical stimuli (200 µs pulse width) delivered via a custom-

made monopolar tungsten electrode (Teflon-insulated to the tip of 50  $\mu\text{m}$  diameter) to the Schaffer collateral-commissural pathway<sup>15</sup>. The circular ROI ( $r = 4$  pixels) was placed into the CA1 stratum radiatum near the stimulation electrode. The ROI also covered a part of the adjacent CA1 stratum pyramidale (Fig. 5A). The intensity of voltage stimulation was adjusted in a manner to produce FDSs with peak amplitudes of ~30% of the highest attainable value.

### **Statistical analysis**

All statistical analyses were performed with SPSS 16.0. When two groups were compared, the student's *t*-test was applied. For three or more group comparisons, one-way or two-way analysis of variance (ANOVA) was performed, followed by Tukey's post-hoc test, as appropriate. All ANOVA *F* and *p* values are reported in supplementary table 1; significant results of the contrast tests are indicated by asterisks in the graphs. Protein-protein associations were analyzed using the Pearson correlation coefficient. *P* values of less than 0.05 were considered significant.

## Reference List

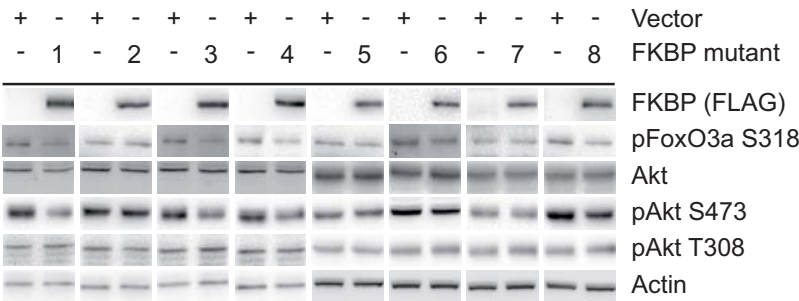
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A

mutants	CoIPs					
	Akt ± SEM	p-value	PHLPP ± SEM	p-value	Hsp90 ± SEM	p-value
1	1	-	1	-	1	-
2	0.95 ± 0.04	0.293	n.d.	n.d.	0.90 ± 0.12	0.450
3	0.98 ± 0.11	0.854	1.10 ± 0.12	0.420	1.08 ± 0.10	0.466
4	0.90 ± 0.07	0.210	0.96 ± 0.05	0.508	n.d.	n.d.
5	n.d.	n.d.	n.d.	n.d.	0.91 ± 0.05	0.162
6	0.60 ± 0.10	0.016	0.59 ± 0.04	0.013	n.d.	n.d.
7	0.50 ± 0.07	0.002	n.d.	n.d.	0.95 ± 0.10	0.679
8	0.90 ± 0.09	0.088	0.90 ± 0.10	0.400	n.d.	n.d.

B



C

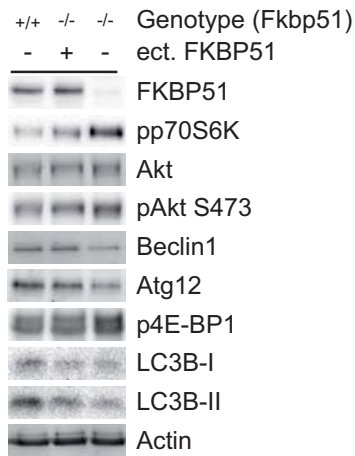
FKBP mutants	pFoxO3a (S318) ± SEM	p-value	pAkt (S473) / Akt ± SEM	p-value	pAkt (T308) / Akt ± SEM	p-value
-	1	-	1	-	1	-
1	0.49 ± 0.14	0.020	0.61 ± 0.10	0.020	1.00 ± 0.22	0.939
2	1.03 ± 0.11	0.821	1.09 ± 0.08	0.307	0.93 ± 0.07	0.334
3	0.50 ± 0.14	0.026	0.54 ± 0.09	0.008	1.24 ± 0.31	0.493
4	0.47 ± 0.13	0.015	0.51 ± 0.13	0.020	1.08 ± 0.23	0.743
5	1.17 ± 0.09	0.143	1.09 ± 0.04	0.123	1.05 ± 0.16	0.758
6	0.73 ± 0.10	0.043	0.73 ± 0.10	0.049	1.14 ± 0.09	0.198
7	1.09 ± 0.07	0.291	1.01 ± 0.09	0.876	1.08 ± 0.20	0.709
8	0.45 ± 0.04	0.013	0.61 ± 0.09	0.014	1.16 ± 0.13	0.151

D

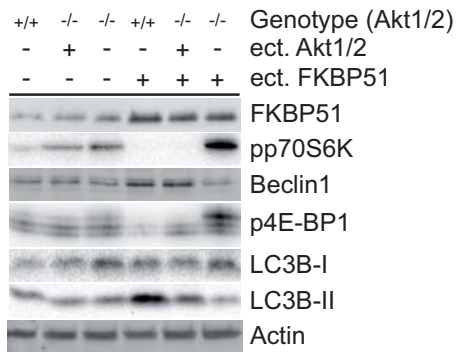
ect. FKBP [ng]	FKBP51 * vector	FKBP52 * vector	FKBP51*FKBP52
25	p = 0.458	p = 0.037	p = 0.701
50	p = 0.046	p = 0.371	p = 0.022
100	p = 0.002	p = 0.255	p < 0.001
150	p < 0.001	p = 0.193	p < 0.001



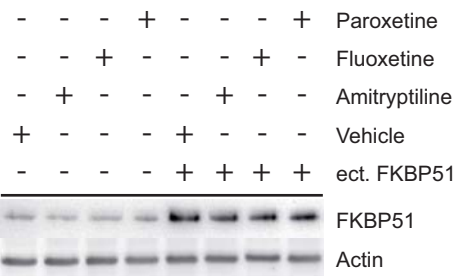
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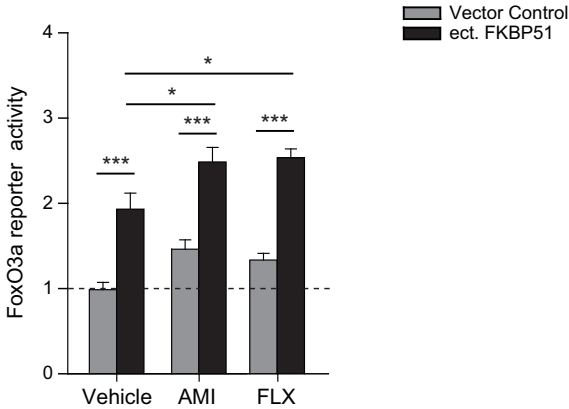
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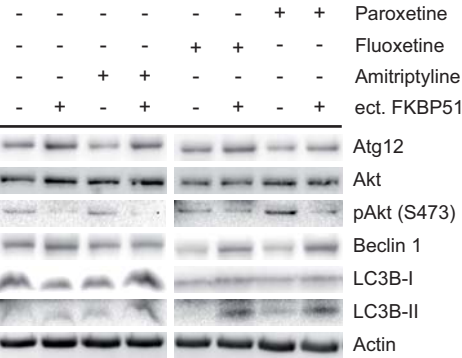
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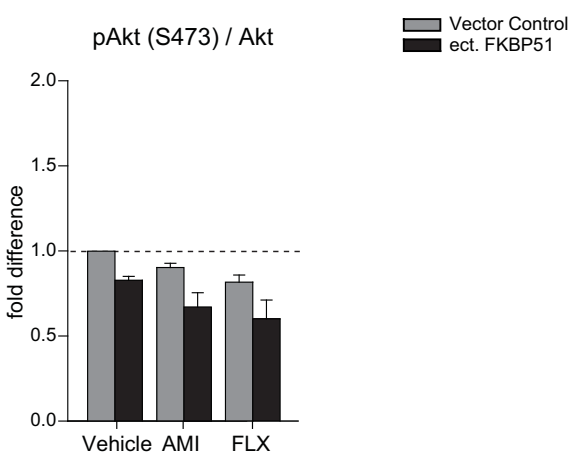
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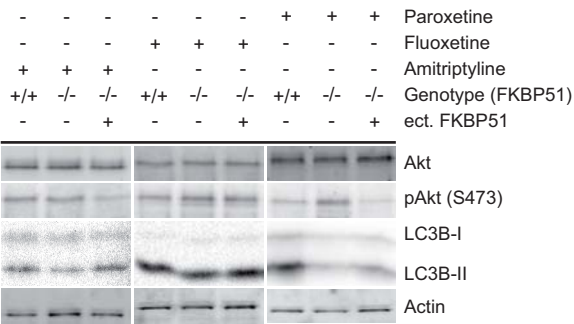
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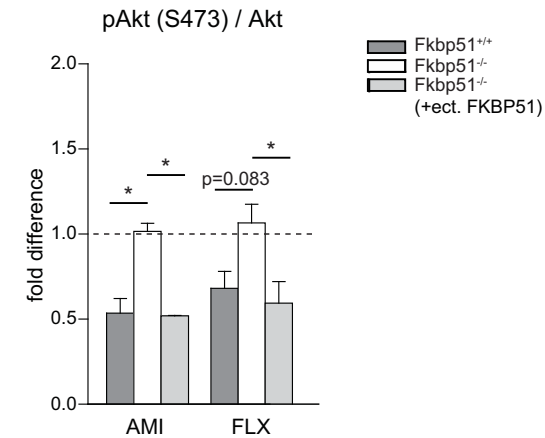
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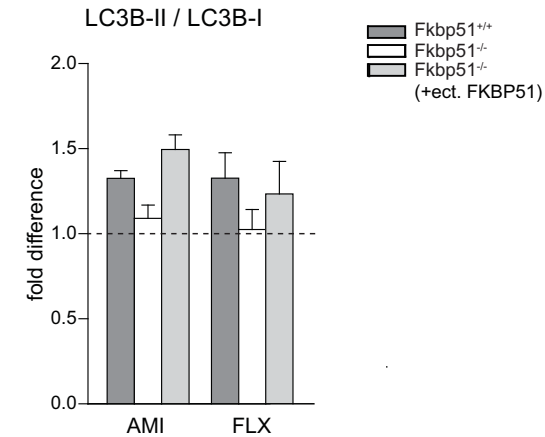
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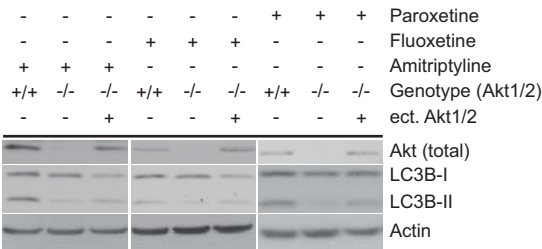
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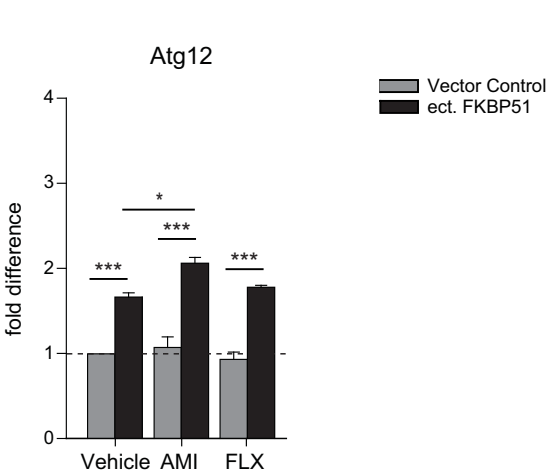
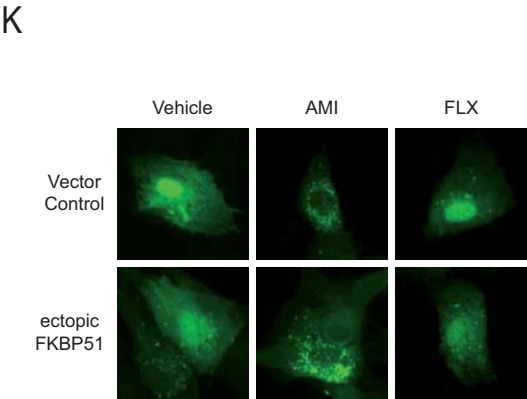
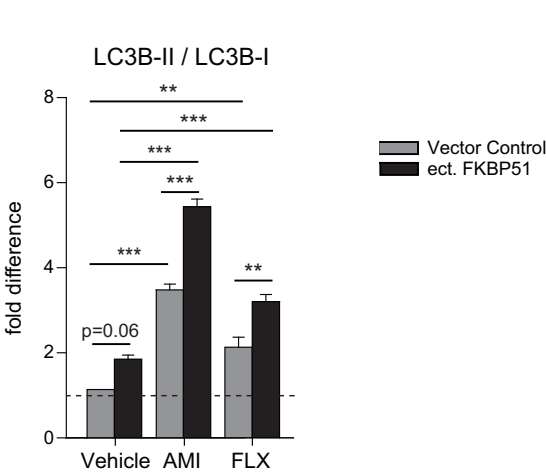
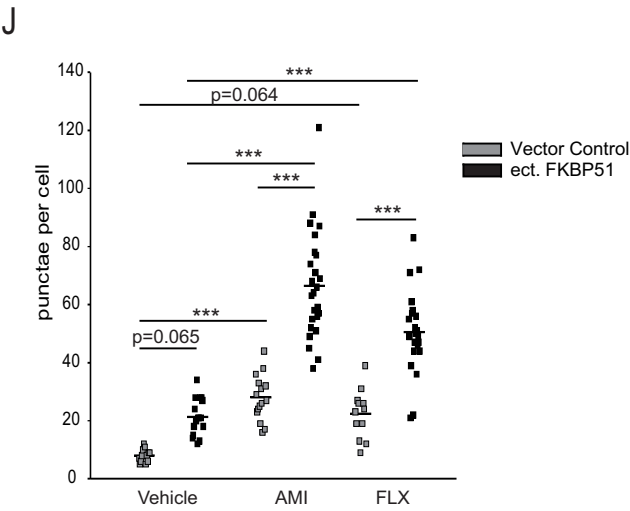
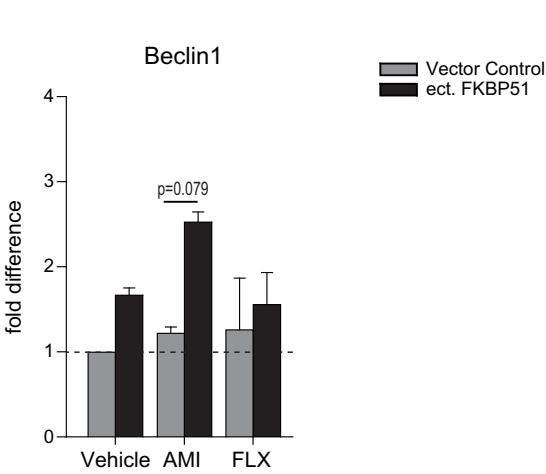
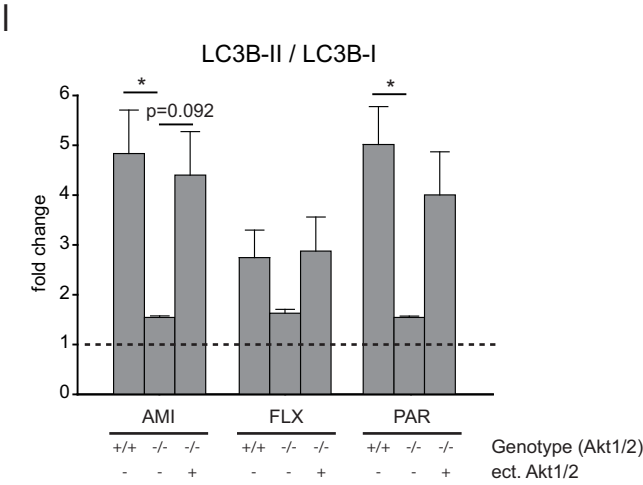


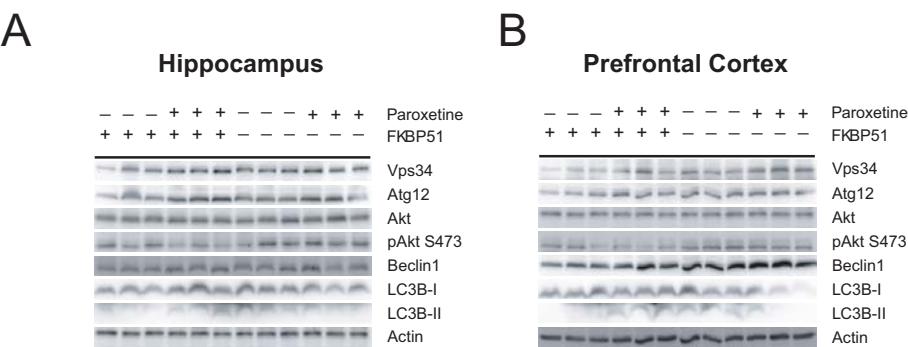
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H







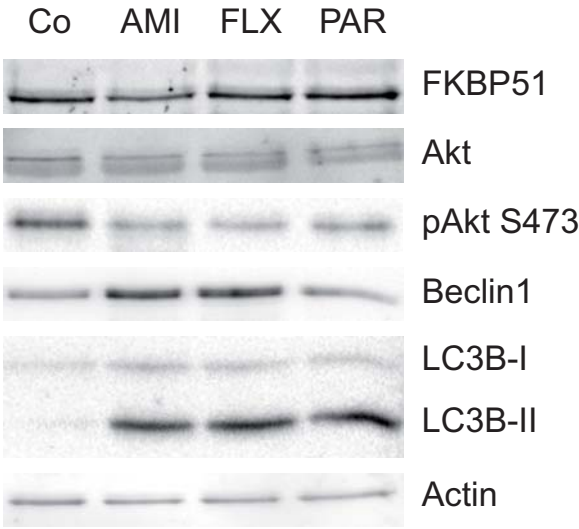


figure	parameter	ANOVA	variable 1		variable 2		variable 1 * variable 2	
2A	pp70S6K	1 Way ANOVA variable 1 = genotype	$F_{2,8} = 3.994$	$p = 0.079$				
2A	p4E-BP1		$F_{2,8} = 17.859$	$p < 0.01$				
2A	pAkt (S473) / Akt		$F_{2,8} = 267.265$	$p < 0.001$				
2A	Beclin 1		$F_{2,8} = 32.996$	$p < 0.001$				
2A	LC3-II / LC3-I		$F_{2,8} = 177.628$	$p < 0.001$				
2A	Atg12		$F_{2,8} = 26.092$	$p < 0.001$				
2B	pp70S6K	2 Way ANOVA variable 1 = treatment variable 2 = genotype	$F_{5,12} = 4.298$	$p = 0.060$	$F_{5,12} = 13.870$	$p < 0.001$	$F_{5,12} = 30.737$	$p < 0.001$
2B	p4E-BP1		$F_{5,12} = 5.470$	$p < 0.05$	$F_{5,12} = 9.474$	$p < 0.01$	$F_{5,12} = 4.997$	$p < 0.05$
2B	Beclin 1		$F_{5,12} = 24.605$	$p < 0.001$	$F_{5,12} = 11.728$	$p < 0.01$	$F_{5,12} = 19.634$	$p < 0.001$
2B	LC3B-II / LC3B-I		$F_{5,12} = 105.288$	$p < 0.001$	$F_{5,12} = 11.107$	$p < 0.01$	$F_{5,12} = 13.721$	$p < 0.001$
3A	FoxO3A reporter activity	2 Way ANOVA variable 1 = treatment variable 2 = genotype	$F_{3,32} = 27.341$	$p < 0.001$	$F_{3,32} = 43.198$	$p < 0.001$	$F_{3,32} = 1.118$	$p = 0.298$
3B	pAkt (S473) / Akt		$F_{3,8} = 126.233$	$p < 0.001$	$F_{3,8} = 203.625$	$p < 0.001$	$F_{3,8} = 12.691$	$p < 0.01$
3C	pAkt (S473) / Akt	1 Way ANOVA variable 1 = genotype	$F_{2,8} = 27.702$	$p < 0.001$				
3D	LC3B-II / LC3B-I		$F_{2,8} = 12.041$	$p < 0.01$				
3E	punctae per cell	2 Way ANOVA variable 1 = treatment variable 2 = genotype	$F_{3,67} = 173.593$	$p < 0.001$	$F_{3,67} = 106.640$	$p < 0.001$	$F_{3,67} = 29.555$	$p < 0.001$
3G	Beclin 1		$F_{2,8} = 135.813$	$p < 0.001$	$F_{2,8} = 389.709$	$p < 0.001$	$F_{2,8} = 41.657$	$p < 0.001$
3H	LC3B-II / LC3B-I		$F_{2,8} = 2785.191$	$p < 0.001$	$F_{2,8} = 357.590$	$p < 0.001$	$F_{2,8} = 111.099$	$p < 0.001$
3I	Atg12		$F_{2,8} = 15.001$	$p < 0.01$	$F_{2,8} = 28.481$	$p < 0.001$	$F_{2,8} = 0.776$	$p = 0.404$
4A	Hip pAkt / Akt		$F_{3,28} = 4.999$	$p < 0.05$	$F_{3,28} = 0.018$	n.s.	$F_{3,28} = 6.508$	$p < 0.05$
4A	PFC pAkt / Akt	2 Way ANOVA variable 1 = treatment variable 2 = genotype	$F_{3,28} = 17.239$	$p < 0.001$	$F_{3,28} = 4.354$	$p < 0.05$	$F_{3,28} = 27.685$	$p < 0.001$
4B	Hip Beclin1		$F_{3,28} = 4.944$	$p < 0.05$	$F_{3,28} = 3.167$	n.s.	$F_{3,28} = 4.299$	$p < 0.05$
4B	PFC Beclin1		$F_{3,28} = 6.335$	$p < 0.5$	$F_{3,28} = 8.827$	$p < 0.01$	$F_{3,28} = 25.002$	$p < 0.001$
4C	Hip LC3B-II / LC3B-I		$F_{3,28} = 2.187$	n.s.	$F_{3,28} = 1.723$	n.s.	$F_{3,28} = 7.967$	$p < 0.01$
4C	PFC LC3B-II / LC3B-I		$F_{3,28} = 28.243$	$p < 0.001$	$F_{3,28} = 16.671$	$p < 0.001$	$F_{3,28} = 32.117$	$p < 0.001$
4D	Hip Atg12		$F_{3,28} = 18.858$	$p < 0.001$	$F_{3,28} = 12.645$	$p < 0.001$	$F_{3,28} = 29.447$	$p < 0.001$
4D	PFC Atg12		$F_{3,28} = 1.942$	n.s.	$F_{3,28} = 5.887$	$p < 0.05$	$F_{3,28} = 9.383$	$p < 0.01$
4E	Hip Vps34		$F_{3,28} = 33.501$	$p < 0.001$	$F_{3,28} = 21.781$	$p < 0.001$	$F_{3,28} = 25.275$	$p < 0.001$
4E	PFC Vps34		$F_{3,28} = 14.334$	$p < 0.001$	$F_{3,28} = 11.231$	$p < 0.01$	$F_{3,28} = 8.125$	$p < 0.01$
4F	immobility		$F_{3,27} = 15.234$	$p < 0.001$	$F_{3,27} = 10.476$	$p < 0.01$	$F_{3,27} = 4.927$	$p < 0.05$
4F	struggling		$F_{3,27} = 25.752$	$p < 0.001$	$F_{3,27} = 13.520$	$p < 0.001$	$F_{3,27} = 4.247$	$p < 0.05$

figure	parameter	ANOVA	variable 1		variable 2		variable 1 * variable 2	
Suppl. 3B	FoxO3A reporter activity	2 Way ANOVA variable 1 = treatment variable 2 = genotype	$F_{5,48} = 9.778$	$p < 0.001$	$F_{5,48} = 99.699$	$p < 0.001$	$F_{5,48} = 0.501$	$p = 0.609$
Suppl. 3D	pAkt (S473) / Akt		$F_{5,12} = 5.781$	$p < 0.5$	$F_{5,12} = 17.236$	$p < 0.001$	$F_{5,12} = 0.129$	$p = 0.880$
Suppl. 3F	pAkt (S473) / Akt		$F_{5,12} = 1.549$	n.s.	$F_{5,12} = 17.786$	$p < 0.001$	$F_{5,12} = 0.156$	$p = 0.858$
Suppl. 3G	LC3B-II / LC3B-I		$F_{5,12} = 1.208$	n.s.	$F_{5,12} = 3.835$	$p = 0.052$	$F_{5,12} = 0.635$	$p = 0.547$
Suppl. 3I	LC3B-II / LC3B-I		$F_{8,18} = 3.244$	$p = 0.063$	$F_{8,18} = 14.749$	$p < 0.001$	$F_{8,18} = 1.139$	$p = 0.370$
Suppl. 3J	punctae per cell		$F_{5,96} = 54.488$	$p < 0.001$	$F_{5,96} = 107.763$	$p < 0.001$	$F_{5,96} = 8.018$	$p < 0.001$
Suppl. 3L	Beclin 1		$F_{5,12} = 1.892$	n.s.	$F_{5,12} = 9.553$	$p < 0.01$	$F_{5,12} = 1.449$	$p = 0.273$
Suppl. 3M	LC3B-II / LC3B-I		$F_{5,12} = 182.414$	$p < 0.001$	$F_{5,12} = 95.458$	$p < 0.001$	$F_{5,12} = 8.243$	$p < 0.01$
Suppl. 3N	Atg12		$F_{5,12} = 6.645$	$p < 0.05$	$F_{5,12} = 205.529$	$p < 0.001$	$F_{5,12} = 2.627$	$p = 0.113$
Suppl. 4C	pAkt (S473) / Akt		$F_{3,10} = 29.227$	$p < 0.001$	$F_{3,10} = 0.135$	n.s.	$F_{3,10} = 0.298$	$p = 0.597$
Suppl. 4C	Beclin 1		$F_{3,10} = 0.893$	n.s.	$F_{3,10} = 0.210$	n.s.	$F_{3,10} = 2.637$	$p = 0.135$

# FK506 Binding Protein 5 Shapes Stress Responsiveness: Modulation of Neuroendocrine Reactivity and Coping Behavior

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**Background:** The Hsp90 cochaperone FK506 binding protein 5 (FKBP5) is an established regulator of the glucocorticoid receptor (GR), and numerous genetic studies have linked it to stress-related diseases such as major depression or posttraumatic stress disorder. However, translational studies including genetic animal models are lacking.

**Methods:** Mice deficient of FKBP5 were generated and analyzed in comparison with wildtype littermates. They were subjected to several test paradigms characterizing their emotionality, stress reactivity, and coping behavior as well as hypothalamus-pituitary-adrenal axis function and regulation. Moreover, protein expression of GR and FKBP5 was determined in different brain structures 8 days after stress exposure. The combined dexamethasone/corticotropin-releasing hormone test was performed both in mice and healthy human subjects of different FKBP5 genotypes. The GR function was evaluated by reporter gene assays.

**Results:** Under basal conditions, deletion of FKBP5 did not change exploratory drive, locomotor activity, anxiety-related behavior, stress-coping, or depression-like behavior. After exposure to different acute stressors of sufficient intensity, however, it led to a more active coping behavior. Moreover, loss of FKBP5 decreased hypothalamus-pituitary-adrenal axis reactivity and GR expression changes in response to stressors. In mice and humans, the FKBP5 genotype also determined the outcome of the dexamethasone/corticotropin-releasing hormone test.

**Conclusions:** This study in mice and humans presents FKBP5 as a decisive factor for the physiological stress response, shaping neuroendocrine reactivity as well as coping behavior. This lends strong support to the concept emerging from human studies of FKBP5 as important factor governing gene–environment interactions relevant for the etiology of affective disorders.

**Key Words:** Dex/CRH test, emotionality, FKBP51, HPA axis, stress-coping behavior, stress reactivity

In the recent years, polymorphisms in the FK506 binding protein 5 (FKBP5), also referred to as FKBP51, have emerged as one of the most important and intriguing associations with stress-related phenotypes and diseases such as major depression and post-traumatic stress disorder (1–7). FKBP5 was originally identified as component of the progesterone receptor-chaperone heterocomplex (8). Its role in stress regulation was first conjectured upon discovery of elevated FKBP5 levels in squirrel monkeys (9,10). These New World primates exhibit glucocorticoid resistance together with markedly elevated levels of plasma cortisol but lack signs of detrimental glucocorticoid excess (11–13). The decreased hormone-binding affinity of their glucocorticoid receptor (GR) (12) was

later largely attributed to the inhibitory action of FKBP5 on GR (14–17).

FKBP5 possesses peptidylprolyl isomerase activity (18) and features a domain for interaction with the central chaperone heat shock protein (Hsp) 90. Although interaction with Hsp90 is essential for its inhibitory action on GR, peptidylprolyl isomerase activity is dispensable (16). Thus, according to the current mechanistic concept, FKBP5 competes with other proteins for access to the Hsp90-GR heterocomplex, thereby interfering with the action of GR-stimulatory factors, in dependency of its expression levels (19).

The well-documented influence of FKBP5 on GR function served as rationale for its inclusion as one of the candidates in the gene association study that first associated *FKBP5* polymorphisms with response to antidepressant treatment (1). Over decades, ample evidence accumulated for an essential role of GR function in stress-related psychiatric disorders such as major depression and post-traumatic stress disorder (20–22). In general, elevated levels of glucocorticoids in response to stressful life events constitute a healthy adaptive reaction, provided this response is balanced and transient. The hypothalamus-pituitary-adrenal (HPA) axis is a key control system to balance hormonal and behavioral responses to stressors. A hallmark of HPA axis regulation is the negative feedback exerted by glucocorticoids via GR on the secretion of stress hormones (20). Substantial evidence suggests that this attenuation of HPA axis activity, which is an integral part of the adaptive response to stressors and challenges, is often impaired in patients suffering from major depression (23,24). For example, major depression has been repeatedly shown to be associated with elevated levels of circulating glucocorticoids, decreased responsiveness to dexa-

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Received Apr 28, 2011; revised Jul 16, 2011; accepted Jul 19, 2011.



methasone (Dex) suppression and increased adrenocortical response to stimulation with corticotropin-releasing hormone (CRH) in the combined Dex/CRH test (20,24). Although the exact physiological consequences of prolonged glucocorticoid elevation are not fully understood (25), it is intriguing that successful treatment of depression mostly goes along with a normalization of HPA axis reactivity. Moreover, remitted individuals with incompletely attenuated HPA axis overdrive have a higher risk of relapse (26). These and other observations led to the formulation of the corticosteroid receptor hypothesis of depression, which stipulates a link between corticosteroid receptor dysfunction and major depression (23).

This hypothesis is further supported by animal experiments (27). For example, mice with impaired GR function due to transgenic antisense RNA expression exhibit neuroendocrine characteristics similar to those observed in major depression, including a hyperactive HPA axis (28). Antidepressant treatment counteracted these alterations (29). In addition, acquired deficit of forebrain GR produces depression-like phenotypes in behavioral and physiological stress reactivity, which were normalized by antidepressant treatment (30).

Given the established role of GR and its regulatory protein FKBP5 in stress-related disorders and associated endophenotypes, we undertook a molecular, neuroendocrine, and behavioral characterization of *Fkbp5*<sup>-/-</sup> mice under basal conditions as well as in response to stressors. We considered the latter point particularly important, because the ability of an organism to respond and the way it responds to stressors have been accepted as crucial factors in stress-related disorders (20). Deletion of *Fkbp5* resulted in alterations of HPA axis reactivity and feedback regulation, induced more active coping behavior, and impacted on the expression changes of GR 8 days after stress exposure. In similarity to the findings in mice, *FKBP5* genotypes in humans also altered the outcome of the Dex/CRH test.

## Methods and Materials

Details of all experimental procedures are provided in Supplement 1. All work was in accord with accepted standards of humane care and use of experimental animals and approved by the appropriate local authority.

### Cell Culture and Reporter Gene Assays

Conditions for cultivating cells, reporter gene assays, and plasmid details have been described previously (19,31–34). Briefly, mouse embryonic fibroblast (MEF) cells derived from knockout (KO) and wildtype (WT) animals were cultured in medium containing steroid-free serum for 24 hours before transfection. Plasmids used were a steroid responsive luciferase reporter and expression vectors for GR and *Fkbp5*. The following day, cells were exposed to Dex and harvested for protein extraction and luciferase activities measured 1 day later.

### Experimental Animals and Housing Conditions

All mice were derived from heterozygous matings. Animals homozygous for the *Fkbp5* KO or WT alleles were used for the experiments. The animal housing and experimental rooms were maintained under standard laboratory conditions. Commercial mouse diet and water were available ad libitum. In all experiments, young adult males were used (10–16 weeks of age). At least 2 weeks before each experiment, mice were single-housed and habituated to the experimental room to avoid transportation and dominance hierarchy effects.

### Basal Behavioral Phenotyping

*Fkbp5*<sup>-/-</sup> and *Fkbp5*<sup>+/+</sup> littermates were tested in paradigms assessing anxiety-related behavior, exploratory drive, locomotor activity, stress-coping, and depression-like behavior (35). The battery of tests consisted of the open-field test, the elevated plus-maze test, the dark-light box test, and the forced swim test (FST). All tests were performed as described previously (36,37) in the order listed between 9:00 AM and 12:00 AM with an inter-test interval of 48 hours (i.e., 1 day rest between the tests, as recommended for successive behavioral testing) (38).

### HPA Axis Function and Regulation

Neuroendocrine stress reactivity (37) was assessed in the same mice that had been characterized in the previously described behavioral test battery by subjecting them to a “stress reactivity test” 1 week after behavioral testing. Briefly, the stress reactivity test comprises a 15-min restraint period and blood sampling immediately before and after stress exposure as well as 75 min thereafter, for determination of corticosterone levels. The test was performed during the first hours of the light phase when corticosterone levels are at the trough of the circadian glucocorticoid rhythm (39,40).

In the Dex/CRH test (41)—assessing HPA axis functions—a blood sample was taken 7 days before the actual test to obtain a basal reference value (“untreated”). On the day of testing, the mice received an IP injection of Dex at 9:00 AM, followed by an injection of CRH at 3:00 PM (.15 mg/kg). Blood samples were collected immediately before CRH injection (“after Dex” value) and 30 min later (“after CRH” value). Two independent Dex/CRH tests were performed with naïve mice, with either a relatively high (2 mg/kg) or low (.05 mg/kg) dose of Dex.

### Dex/CRH Test and Genotyping in Human Subjects

Healthy subjects between 20 and 44 years of age (33 men, 32 women; mean age 27.4 years, SD 6.5, all Caucasians from German descent) without any history of psychiatric or severe somatic disorders, verified by standardized clinical interviews, were recruited from webpage advertising and local notice boards. Participants gave written informed consent after all study details were explained. The study protocol was approved by the ethical committee at the Medical Department of the Ludwig-Maximilians-University Munich, Germany. Subjects received 1.5 mg Dex orally at 11 PM and were injected with human CRH (100 µg) at 3 PM the next day. Blood samples for determination of cortisol levels were drawn 1 day before Dex treatment, immediately before CRH injection, and 30 min thereafter.

*FKBP5* genotyping was performed with pyrosequencing. The single nucleotide polymorphism rs1360780 was selected, because we found it associated with *FKBP5* expression changes (1). Genotype call rate was 100%, and no deviation from the Hardy-Weinberg-Equilibrium was observed ( $p = .358$ ).

### Effects of Acute Stressors on Coping Behavior

In order to investigate the effects of acute stress exposure on the coping behavior of *Fkbp5*<sup>-/-</sup> and *Fkbp5*<sup>+/+</sup> mice, several experiments were performed with stressors of different intensity and addressing the response of the animals in the FST 24 hours later.

### Tissue Protein Extraction and Immunoblotting

Mice were sacrificed under basal conditions 8 days after the last behavioral test or the Dex/CRH tests, and total protein was extracted from different brain regions. Immunoblots were performed as described with minor modifications (16). Briefly, equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a membrane, probed with suitable pri-

mary and secondary antibodies, and visualized by chemoluminescence. For quantification, measurement of the optical densities of immunoblot bands (with  $\beta$ -actin or glyceraldehyde-3-phosphate dehydrogenase as loading controls) was performed with ImageJ software.

### Statistics

Because a normal distribution and variance homogeneity could not always be assumed, data were analyzed with nonparametric or parametric statistics where appropriate. All tests were applied two-tailed. For the behavioral, neuroendocrine, and protein expression data of mice, two independent samples were compared with the Mann-Whitney *U* test (MWU-test) or the Student *t* test. More than two independent samples were compared with analysis of variance, followed by Bonferroni corrected post hoc tests. For all tests, we considered  $p < .1$  as a trend (T) and  $p < .05$  as statistically significant.

Genetic *FKBP5* effects on Dex suppressed baseline cortisol and on the cortisol response to CRH in human subjects was calculated with a permutation test after correcting for the effects of age and gender. Empirical *p* values of the C-carrier model (representing the rs1360780 genotype for low vs. high *FKBP5* lymphocyte protein concentrations) are reported with the parameter-free permutation-based approach with 100,000 runs.

## Results

### Deletion of *FKBP5* Causes Mild GR Hypersensitivity

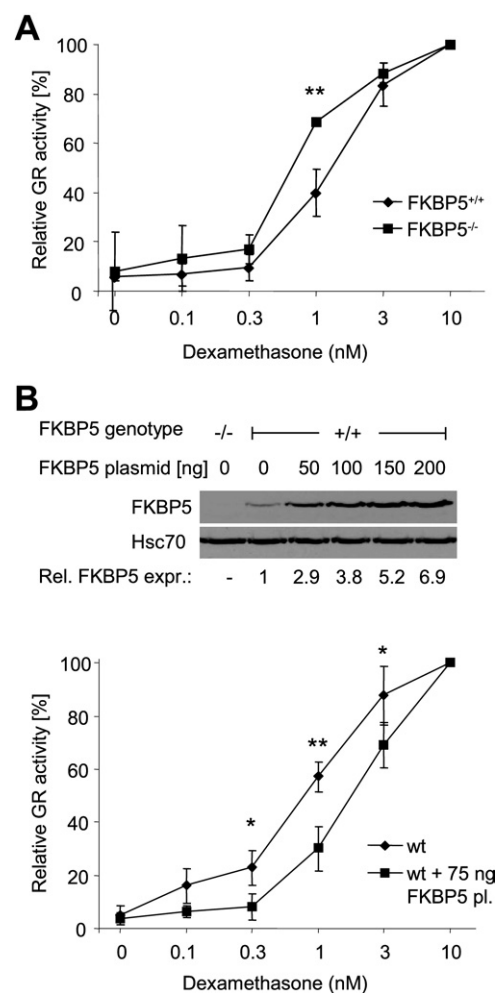
Because *FKBP5* is an established inhibitor of GR hormone binding activity (15,16), its deletion (described in Supplement 1) is expected to lead to a higher hormone sensitivity of GR, if no compensatory mechanisms are elicited. To test this in a defined system, we performed GR-dependent reporter gene assays in MEF cells derived from KO and WT animals. In *Fkbp5*<sup>-/-</sup> cells, GR exhibited a slightly increased responsiveness of its transcriptional activity (Figure 1A). We further addressed in MEF cells that WT animals react with an acute elevation of *FKBP5* levels upon glucocorticoid exposure (42,43), whereas KO animals are deprived of this regulatory feedback loop. This should increase the difference in hormone responsiveness, unless *FKBP5* expression in WT is already at high levels such that a further increase would not change GR activity. We calibrated ectopic expression of *FKBP5* to yield an approximately threefold increase of total *FKBP5* and observed a further decrease of GR hormone responsiveness (Figure 1B).

### Loss of *FKBP5* Shows No Effect on Emotional Behavior Under Basal Conditions

To assess potential effects of *FKBP5* deletion and the ensuing mild GR hyperactivity, we applied a battery of tests characterizing the behavior of *Fkbp5*<sup>-/-</sup> and *Fkbp5*<sup>+/-</sup> mice under basal conditions. No significant differences were revealed between the two groups in all parameters assessed in the open-field test, the elevated plus-maze test, the dark-light box test, and the FST (Table S1 in Supplement 1). Animals of both genotypes displayed comparable levels of anxiety-related behavior, exploratory drive, locomotor activity, stress-coping, and depression-like behavior, when tested under nonstressed conditions.

### *FKBP5* Modulates HPA Axis Function and Regulation in Mice and Men

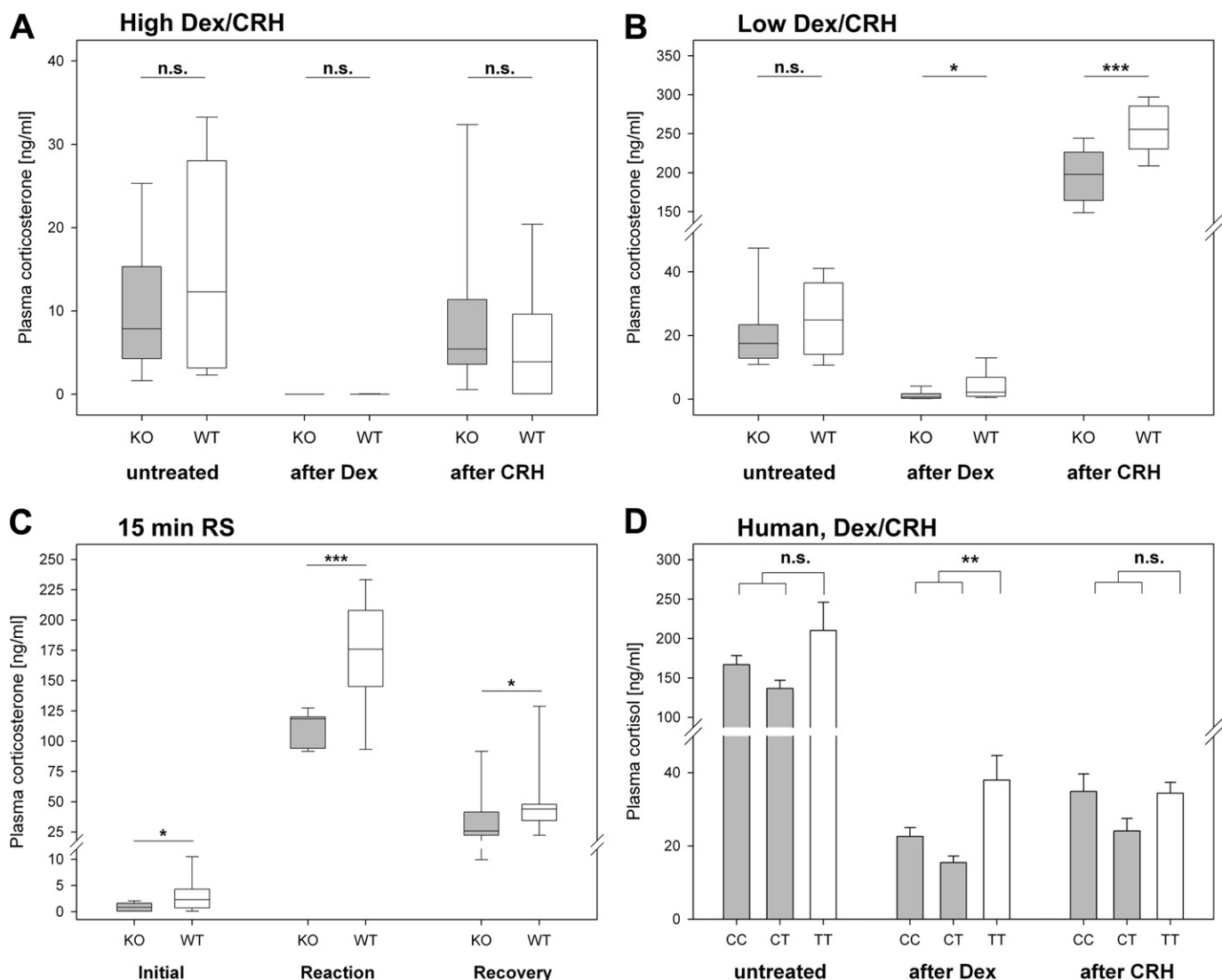
**Dex/CRH Tests in Mice.** To more directly assess potential consequences of the moderate difference in GR function between *Fkbp5*<sup>-/-</sup> and *Fkbp5*<sup>+/-</sup> mice on the neuroendocrine level, we performed two independent Dex/CRH tests, applying either a relatively high or a relatively low dose of Dex.



**Figure 1.** Loss of FK506 binding protein 5 (*FKBP5*) changes glucocorticoid receptor (GR) responsiveness. **(A)** Reporter gene assays in mouse embryonic fibroblast (MEF) cells derived from *Fkbp5* knockout and wildtype mice were transfected with the reporter, GR, and control plasmids and treated with hormone as indicated. **(B)** Western panels: MEF wildtype cells were transfected with additional *FKBP5* expression vector to mimic stress-induced increase of *Fkbp5*. Expression levels were determined relative to heat shock constitutive 70 and quantified. *FKBP5* (75 ng) expression plasmid was used in the reporter gene assays in addition to the plasmids used in **A**. Reporter results in **A** and **B** represent data from 3 to 5 experiments performed in triplicate; the activity at the highest hormone concentration was set to 100. Data are given as means and SEM. Statistically significant differences are indicated (\* $p < .05$ , \*\* $p < .01$ ; Student *t* test, panel **A**, at 1 nmol/L dexamethasone (Dex):  $t = 5.03$ ,  $p = .007$ ; panel **B**, at .3 nmol/L Dex:  $t = 2.95$ ,  $p = .026$ , at 1 nmol/L Dex:  $t = 5.53$ ,  $p = .002$ ; at 3 nmol/L Dex,  $t = 2.99$ ,  $p = .024$ ).

In both tests, animals of both genotypes responded to the injection of Dex with clearly decreased plasma corticosterone concentrations (Figures 2A and 2B). The high dose of Dex strongly suppressed adrenocortical activity in *Fkbp5*<sup>-/-</sup> as well as *Fkbp5*<sup>+/-</sup> mice, so that corticosterone levels were below the detection limit and did not differ between the genotypes (after Dex value, Figure 2A). When treated with the lower dose of Dex, however, *FKBP5*-deficient mice showed a stronger suppression of corticosterone secretion compared with their WT littermates (after Dex value, Figure 2B).

To the stimulation with CRH, all mice responded with an increased secretion of glucocorticoids (Figures 2A and 2B). In the high-dose Dex experiment, corticosterone concentrations were



**Figure 2.** The *Fkbp5* genotype affects hypothalamus-pituitary-adrenal axis function as revealed by stress reactivity and Dex/corticotropin-releasing hormone (CRH) testing. **(A, B)** Combined Dex/CRH test in mice. Corticosterone response to a pharmacological suppression of adrenocortical activity with either a relatively high (2 mg/kg, panel **A**) or relatively low (.05 mg/kg, panel **B**) dose of Dex and a subsequent stimulation with CRH. Plasma corticosterone concentrations were measured in male *Fkbp5* knockout (KO) and wildtype (WT) mice 1 week before (untreated) and 6 hours after Dex treatment (after Dex) and 30 min after CRH injection (after CRH). Data are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes), as well as 10% and 90% percentiles (whiskers). Statistical differences between the groups are indicated ( $p > .1$  nonsignificant (n.s.),  $*p < .05$ ,  $***p < .001$ ) above the columns for each time point (panel **A**, high-dose Dex, Mann-Whitney *U* test (MWU-test),  $N_{KO} = 10$ ,  $N_{WT} = 10$ , untreated:  $U = 57$ ,  $p = .386$ , after Dex:  $U = 34.5$ ,  $p = .103$ , after CRH:  $U = 35$ ,  $p = .256$ ; panel **B**, low-dose Dex, MWU-test,  $N_{KO} = 12$ ,  $N_{WT} = 12$ , untreated:  $U = 40$ ,  $p = .450$ , after Dex:  $U = 28$ ,  $p = .011$ , after CRH:  $U = 14$ ,  $p = .001$ ). **(C)** Stress reactivity testing. Plasma corticosterone concentrations immediately before (initial) and after (reaction) a 15-min restraint (RS) period as well as 75 min after termination of the stressor (recovery) in male *Fkbp5* KO and WT mice. Data and statistical differences are given as described above (panel **C**, MWU-test,  $N_{KO} = 11$ ,  $N_{WT} = 13$ , initial:  $U = 34$ ,  $p = .028$ , reaction:  $U = 14$ ,  $p < .001$ , recovery:  $U = 36$ ,  $p = .040$ ). **(D)** Dex/CRH test in humans. Graph depicts the cortisol response to the Dex/CRH test combining a pharmacological suppression of adrenocortical activity with a relatively low dose of Dex (1.5 mg) and a subsequent stimulation with CRH (100  $\mu$ g) in healthy human subjects. Plasma cortisol concentrations were measured in C allele carriers (CC) ( $n = 34$ ; CT,  $n = 23$ ) and in noncarriers (TT) ( $n = 7$ ) of the *FKBP5* single nucleotide polymorphism rs1360780 1 day before (untreated) and 16 hours after Dex treatment (after Dex) and 30 min after CRH injection (after CRH). Data are given as means and SEM. Statistical differences between the genotypes (recessive model) are indicated ( $p > .1$  n.s.,  $**p < .01$ ) above the columns for each time point (permutation tests with 100,000 permutations, performed with age- and gender-corrected residuals, untreated:  $p = .101$ , after Dex:  $p = .004$ , after CRH:  $p = .898$ ). Other abbreviations as in Figure 1.

only slightly elevated 30 min after CRH injection (nearly reaching the levels observed before Dex treatment) and did not differ between *Fkbp5*<sup>-/-</sup> and *Fkbp5*<sup>+/+</sup> mice (after CRH value, Figure 2A). In the low-dose Dex experiment, however, the CRH injection induced a robust increase of corticosterone concentrations, which was significantly less pronounced in *Fkbp5*<sup>-/-</sup> mice than in their *Fkbp5*<sup>+/+</sup> littermates (after CRH value, Figure 2B).

The corticosterone concentrations measured under basal conditions at 3:00 PM 1 week before the Dex/CRH test did not differ significantly between *Fkbp5*<sup>-/-</sup> and *Fkbp5*<sup>+/+</sup> mice either in the low-dose Dex experiment (untreated value, Figure 2B) or in the high-dose Dex experiment (untreated value, Figure 2A).

**Stress Reactivity Testing in Mice.** To further test the reactivity (and recovery) of the HPA axis in a more physiological paradigm, we

used a 15-min restraint period as moderate psychological stressor. Compared with *Fkbp5*<sup>+/+</sup> mice, plasma corticosterone concentrations were significantly lower in *Fkbp5*<sup>-/-</sup> mice at all three time points (Figure 2C) (i.e., immediately before [initial value], directly after [reaction value], and 75 min after termination of the stressor [recovery value]).

**Dex/CRH Test in Humans.** To relate the findings on HPA axis function and regulation in our mouse model to the situation in humans, we performed the Dex/CRH test in 65 genotyped volunteers screened for absence of mental and severe physical disorders during lifetime. Individuals whose genotype previously had been associated with lower levels of FKBP5 (rs1360780 C-carriers) reacted with a stronger suppression of cortisol after Dex treatment (Figure 2D). Although this finding is consistent with the difference observed in mice, we did not observe a genotype-dependent difference in overall cortisol levels after stimulation with CRH.

### FKBP5<sup>-/-</sup> Mice Exhibit More Active Coping Behavior After Stress Exposure

Stressors of different intensity were used to investigate the effects of acute stress exposure on the coping behavior of *Fkbp5*<sup>-/-</sup> and *Fkbp5*<sup>+/+</sup> mice in the FST 24 hours later. In a first experiment, we exposed mice to a 15-min restraint stressor, returned them to their home cage for 15 min, and then subjected them to a 6-min FST. In this FST, *Fkbp5*<sup>-/-</sup> animals showed a trend toward more active coping with the aversive situation (i.e., more swimming behavior was observed compared with their WT littermates) (Figure 3A). The second FST, which was performed 24 hours after the combined restraint and first FST stressor, revealed more pronounced statistically significant behavioral differences (Figure 3B). This experiment was also replicated with an independent batch of animals, yielding very similar results (Table S2 in Supplement 1).

In a second experiment with reduced overall stress intensity, the *Fkbp5*<sup>-/-</sup> and *Fkbp5*<sup>+/+</sup> animals were subjected to the 15-min restraint stressor, but without FST testing shortly afterward. In this experiment, no differences in coping behavior between the two groups were observed during the FST performed 24 hours after stress exposure (Figure 3C).

In another experiment with increased stress intensity, *Fkbp5*<sup>-/-</sup> and *Fkbp5*<sup>+/+</sup> mice were subjected to a prolonged restraint period of 60 min and tested in the FST 24 hours later. The FKBP5-deficient mice again exhibited more swimming and less floating behavior than WT animals (Figure 3D) (i.e., showed more active coping).

Confirming our assumption that 60-min restraint constitutes a stronger stressor than 15-min restraint, the prolonged restraint elicited a higher increase of corticosterone concentrations immediately after releasing the mice. Importantly, the genotypic differences in HPA axis reactivity were similar in both experiments (Figures 2C and 3E), with FKBP5-deficient mice showing significantly lower endocrine stress reactivity.

### Deletion of FKBP5 Impacts on Stress-Induced Changes of Protein Expression

We compared the expression levels of GR between WT and KO animals 8 days after exposure to the Dex/CRH tests (Figure 2) or after the combined exposure to the restraint (15 min or 60 min) and FST (Figure 3). In the Dex/CRH tests, only the experiment with the lower dose of Dex produced a difference between the genotypes, with the WT animals exhibiting lower levels of GR expression in the hippocampus, prefrontal cortex, and cerebellum (Figures 4A and B).

For the combined restraint and FST exposure, only the experiment with 60-min restraint resulted in a significant change of hip-

pocampal GR expression, where WT but not KO animals exhibited reduced GR expression compared with unstressed control animals (Figure 4C). In the WT mice of this experiment, we also assessed FKBP5 protein levels in the hippocampus and observed a small but significant decrease of FKBP5 expression eight days after stress exposure (Figure 4D). Similar results were obtained for the prefrontal cortex and the cerebellum (data not shown).

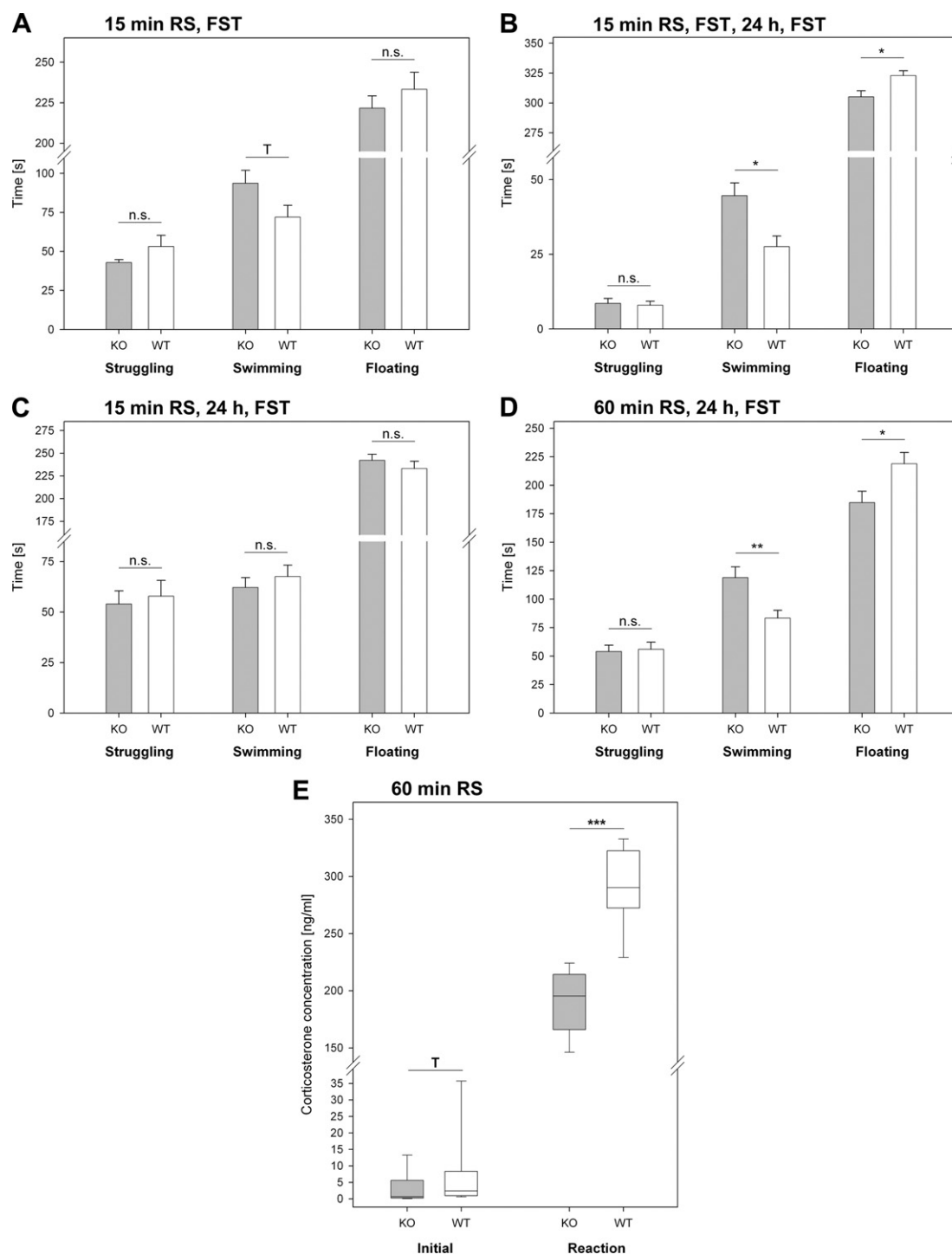
## Discussion

It has been recognized over the years that decisive for the development of stress-related diseases such as major depression is not a particular traumatic event per se or a particular genetic endowment but the interplay of genetic and environmental factors. Therefore, it is crucial for increasing our understanding of affective disorders to investigate how individual differences in stress responsiveness arise and by which mechanisms an organism is able to cope with challenges and stressful situations. We report here that *Fkbp5* gene deletion results in a mild GR hypersensitivity, changes in neuroendocrine reactivity and feedback regulation of the HPA axis, a more active coping behavior after exposure to stressors, and alterations in the stress-induced changes of GR expression. Similarly, the *FKBP5* genotypes in humans shown to be associated with different *FKBP5* expression levels turned out to be linked to the outcome of the Dex/CRH test. The functional link between FKBP5 and GR most likely forms the basis for these effects, although the possibility exists that other important actions of FKBP5 (19,44–46) might contribute to the observed endophenotypes.

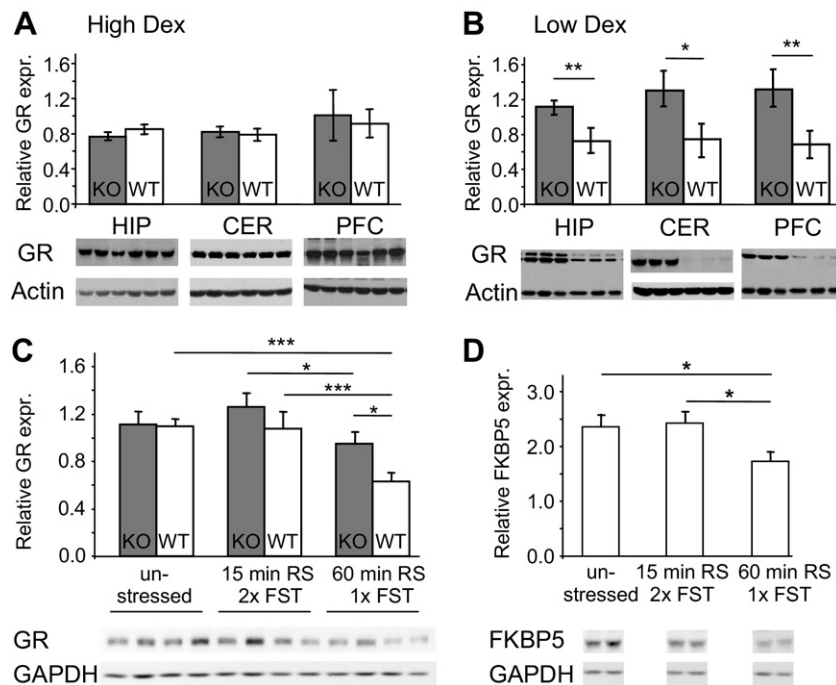
In general, gene KO strategies carry the risk of compensatory molecular reactions that compromise detection of protein function. Because FKBP5 acts on GR via its interaction with Hsp90 (16), other inhibitory factors competing for Hsp90 binding could in principle take over the function of attenuating GR activity (19). Our experiments, however, indicate a more sensitive GR in *Fkbp5*-deleted MEF cells (Figure 1A), which was also supported by hormone binding experiments in brain extracts (not shown). Thus, other Hsp90 cochaperones apparently did not compensate for the loss of FKBP5 (e.g., by decreasing FKBP4) (Figure 1B). An enhanced GR sensitivity in animals devoid of FKBP5 is the most likely explanation for the changes in HPA axis settings observed here. This includes the lower levels of basal morning corticosterone (Figures 2 and 3E) and particularly the reduced secretion of corticosterone in response to pharmacological or psychological stressors (Figures 2C and 3E). This interpretation is corroborated by a recent report of a gain of function GR knock-in mouse (47). In that study, the endogenous GR was replaced by the human GR<sub>M604L</sub> mutant, which is activated at lower glucocorticoid concentrations than wildtype GR (48). Those mice were phenotypically normal under basal conditions—with birth weight, postnatal growth, and development indistinguishable from WT littermates—but exhibited changes in HPA axis activity (47), in similarity to our findings in the *Fkbp5*<sup>-/-</sup> mice presented here.

Our observation that the Dex/CRH test revealed a genotype difference only at the lower dose of Dex might seem surprising at first (Figure 2). This dose-dependency most likely reflects the shift of GR responsiveness toward lower concentrations of hormone, which was observed here (Figure 1) and elsewhere (15,16). The higher dose of Dex that did not produce a genotype effect presumably amounted to a saturating concentration of hormone that activates GR irrespectively of the FKBP5 status. In contrast, the fortyfold lower dose of Dex that exposed a genotype effect presumably resulted in sub-saturating hormone concentration, as evidenced by the less pronounced suppression of corticosterone (Figure 2), a





**Figure 3.** Fkbp5 modulates coping behavior after stress exposure. **(A, B)** Coping behavior of *Fkbp5* KO and WT mice was assessed in the forced swim test (FST) performed shortly after exposure to a 15-min RS stressor **(A)** and 24 h after the first FST **(B)**. Data are given as means and SEM. Statistical differences between the groups are indicated ( $p > .1$  n.s.,  $p < .1$  trend (T),  $*p < .05$ ,  $**p < .01$ ) in both panels above the columns for each behavioral pattern (panel **A**, first FST, MWU-test,  $N_{KO} = 11$ ,  $N_{WT} = 11$ , struggling:  $U = 46$ ,  $p = .341$ , swimming:  $U = 34$ ,  $p = .082$ , floating:  $U = 48$ ,  $p = .412$ ; panel **B**, second FST, MWU-test,  $N_{KO} = 11$ ,  $N_{WT} = 11$ , struggling:  $U = 56$ ,  $p = .768$ , swimming:  $U = 22$ ,  $p = .011$ , floating:  $U = 24$ ,  $p = .017$ ). **(C, D)** Coping behavior of male KO and WT mice was assessed in the FST 24 hours after either 15-min **(C)** or 60-min **(D)** of RS stress. Data and statistical differences are given as described above (panel **C**, MWU-test,  $N_{KO} = 11$ ,  $N_{WT} = 11$ , struggling:  $U = 46$ ,  $p = .341$ , swimming:  $U = 34$ ,  $p = .082$ , floating:  $U = 48$ ,  $p = .412$ ; panel **D**, MWU-test,  $N_{KO} = 12$ ,  $N_{WT} = 12$ , struggling:  $U = 71$ ,  $p = .954$ , swimming:  $U = 27$ ,  $p = .009$ , floating:  $U = 31$ ,  $p = .018$ ). **(E)** Plasma corticosterone concentrations immediately before (initial) and after (reaction) the 60-min RS period (c.f. panel **D**) were determined. Data are given as box plots showing medians (lines in the boxes), 25% and 75% percentiles (boxes) as well as 10th and 90th percentiles (whiskers). Statistical differences between the groups are indicated ( $p < .1$  T,  $***p < .001$ ) above the columns for each time point (MWU-test,  $N_{KO} = 12$ ,  $N_{WT} = 12$ , initial:  $U = 41$ ,  $p = .073$ , reaction:  $U = 3$ ,  $p < .001$ ). Other abbreviations as in [Figures 1 and 2](#).



**Figure 4.** FKBP5 modulates stress-induced changes of protein expression. **(A, B)** GR protein levels in the bilaterally pooled hippocampi (HIP), cerebellum (CER), and prefrontal cortex (PFC) were determined 8 days after completion of the combined Dex/CRH tests (cf. Figure 2) by immunoblotting and normalized to actin levels (panel A: high-dose Dex, panel B: low-dose Dex). Brains from seven male mice for each genotype and condition were extracted, and immunoblots were performed in triplicates. Depicted bands on the immunoblots represent samples from six individual mice, corresponding to the conditions displayed in the graphs above. Data are given as means and SEM. Statistical differences between the groups are indicated (\* $p < .05$ , \*\* $p < .01$ ) above the columns for each brain region (panel A, high-dose Dex, Student  $t$  test,  $N_{KO} = 7$ ,  $N_{WT} = 7$ , HIP:  $t = .651$ ,  $p = .161$ , CER:  $t = .765$ ,  $p = .215$ , PFC:  $t = .717$ ,  $p = .343$ ; panel B, low-dose Dex, Student  $t$  test,  $N_{KO} = 7$ ,  $N_{WT} = 7$ , HIP:  $t = 1.76$ ,  $p = .008$ , CER:  $t = 1.98$ ,  $p = .034$ , PFC:  $t = 1.85$ ,  $p = .007$ ). **(C)** GR protein levels in the bilaterally pooled HIP were determined in six *Fkbp5* KO and six WT littermates 8 days after the stress experiments presented in Figures 3B and 3D (i.e. either 15-min RS followed by two FST or 60-min RS followed by one FST on the next day or unstressed control subjects [six mice of each genotype left undisturbed in their home cages]). Immunoblot experiments were performed in triplicates, and GR signals were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. The blot depicted shows 12 adjacent bands with 2 bands representative for each experimental group, respectively. Statistical analysis employing two-way analysis of variance revealed significant group differences for treatment [ $F(2,30) = 14.4$ ,  $p < .001$ ] and *Fkbp5* genotype [ $F(1,30) = 7.8$ ,  $p = .009$ ]. The interaction of both factors was not significant ( $p = .150$ ). **(D)** FKBP5 protein levels were determined in the same six WT animals used for determination of GR in panel C with the same procedure. Blots depicted show two adjacent immunoblot bands each and constitute representative examples. One-way analysis of variance revealed significant group differences for treatment [ $F(2,15) = 6.7$ ,  $p = .009$ ]. Significant differences in the pairwise Bonferroni post hoc tests are indicated above the respective columns (\* $p < .05$ , \*\*\* $p < .001$ ). Other abbreviations as in Figures 1 and 2.

condition in which the dependency of GR activity on FKBP5 expression is most pronounced (15,16). Notably, the differences we observed in response to the lower dose of Dex between FKBP5-expressing and nonexpressing mice are paralleled in humans, where the genotype previously shown to be associated with lower levels of FKBP5 (1) also exhibited lower levels of cortisol after Dex treatment (Figure 2).

Expression changes of genes relevant to the function of the HPA axis have been frequently described to be associated with stress exposure—in particular stressful events during sensitive periods of early development (49). For example, in rats, lower levels of maternal care led to decreased GR expression in the hippocampus of the offspring (50). Maternal separation led to decreased expression of GR in the hippocampus and forebrain (51,52), apparently in a time-dependent fashion, because it is preceded by an increase of GR expression (51). Exposure of pregnant rats to Dex also caused GR expression changes in the offspring, which was dependent on the brain region and duration/timing of treatment (53). In humans, a postmortem analysis of suicide victims revealed an association of childhood abuse with decreased hippocampal GR expression (54). Because there is more evidence linking GR expression to psychopathological conditions (20,55), there are intense research efforts aiming at understanding the mechanisms governing programming

of GR. Epigenetic mechanisms are very likely to contribute (54,56,57), and our findings of a genotypic difference in GR expression after stress exposure (Figure 4) put FKBP5 forward as a candidate to contribute to stress-induced GR expression changes. Our observation that the genotypic difference was dependent on the dose/intensity of the pharmacological/psychological stressor most likely relates to the hormone responsiveness of GR, where differences were exposed at sub-saturating concentrations of steroids but barely at concentrations above and below.

It is worth mentioning that our study was limited to male mice. Females were not included, due to methodological difficulties of monitoring the estrous cycle in a noninvasive manner, which is mandatory to avoid systematically biasing the results. Nevertheless, it will be important to address a potential gender difference in the effect of FKBP5 in preclinical models, because prevalence rates of major depression are higher in women (58). Actually, one clinical study suggests a gender difference in the FKBP5-genotype association with major depression (59).

The effect of FKBP5 deletion on HPA axis parameters discussed in the preceding text can be interpreted as a compensatory reaction to altered GR sensitivity restoring an adequate balance of receptor and hormone and therefore should not have further physiological consequences. Accordingly, we did not find genotype-

dependent behavioral differences under basal conditions. However, by deleting *Fkbp5*, the animals are deprived not simply of a static factor that calibrates GR activity but also of the ultra-short intracellular feedback loop, a peculiar hallmark of molecular stress regulation linking FKBP5 and GR. This feedback loop leads to an increase of FKBP5 upon GR activation (60,61), thereby limiting extended, excessive activation of GR. The lack of this feedback loop in *Fkbp5*<sup>−/−</sup> animals might be the basis for our observation that phenotypic differences in coping behavior during the FST are exposed only if preceded by stressors of sufficient strength (Figure 3). Very recently, this was similarly shown for anxiety-related behavior (62), further corroborating the relevance of the *Fkbp5*<sup>−/−</sup> mouse model.

In this respect, it is intriguing that several studies in humans attest to the concept that FKBP5 is decisive for the development of stress-related mental disorders only in combination with traumatic events (2,63–65). We hypothesize that, taken together with our results in mice, the reported polymorphisms are linked—directly or indirectly—to the ultra-short feedback loop connecting GR and FKBP5 as a crucial component of stress regulation. Among the possible mechanisms, genetic polymorphisms might change programming of FKBP5 expression, because it has been shown recently that FKBP5 is amenable to epigenetic regulation (43). In conclusion, our findings translate clinical observations on FKBP5 into animal-based experimental systems. This should encourage more detailed studies testing the hypothesis that genotype-dependent reaction of FKBP5 expression to stressful life events determines the risk for development of affective disorders, ultimately leading to improved treatment regimes.

*This study was supported by the Max Planck Society and the Horst Kübler foundation, Bad Ragaz. Drs. Holsboer and Rein are co-inventors of the following pending patent application: FKBP5: a novel target for antidepressant therapy (International publication number: WO 2005/05450). We are indebted to Sergej Asmus, Bozidar Novak, Kathrin Hafner, Lisa Tietze, and Albin Varga for technical assistance. The other authors report no biomedical financial interests or potential conflicts of interest.*

*Supplementary material cited in this article is available online.*

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# Differential Impact of Tetratricopeptide Repeat Proteins on the Steroid Hormone Receptors

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## Abstract

**Background:** Tetratricopeptide repeat (TPR) motif containing co-chaperones of the chaperone Hsp90 are considered control modules that govern activity and specificity of this central folding platform. Steroid receptors are paradigm clients of Hsp90. The influence of some TPR proteins on selected receptors has been described, but a comprehensive analysis of the effects of TPR proteins on all steroid receptors has not been accomplished yet.

**Methodology and Principal Findings:** We compared the influence of the TPR proteins FK506 binding proteins 51 and 52, protein phosphatase-5, C-terminus of Hsp70 interacting protein, cyclophilin 40, hepatitis-virus-B X-associated protein-2, and tetratricopeptide repeat protein-2 on all six steroid hormone receptors in a homogeneous mammalian cell system. To be able to assess each cofactor's effect on the transcriptional activity of on each steroid receptor we employed transient transfection in a reporter gene assay. In addition, we evaluated the interactions of the TPR proteins with the receptors and components of the Hsp90 chaperone heterocomplex by coimmunoprecipitation. In the functional assays, corticosteroid and progesterone receptors displayed the most sensitive and distinct reaction to the TPR proteins. Androgen receptor's activity was moderately impaired by most cofactors, whereas the Estrogen receptors' activity was impaired by most cofactors only to a minor degree. Second, interaction studies revealed that the strongly receptor-interacting co-chaperones were all among the inhibitory proteins. Intriguingly, the TPR-proteins also differentially co-precipitated the heterochaperone complex components Hsp90, Hsp70, and p23, pointing to differences in their modes of action.

**Conclusion and Significance:** The results of this comprehensive study provide important insight into chaperoning of diverse client proteins via the combinatorial action of (co)-chaperones. The differential effects of the TPR proteins on steroid receptors bear on all physiological processes related to steroid hormone activity.

**Citation:** Schülke J-P, Wochnik GM, Lang-Rollin I, Gassen NC, Knapp RT, et al. (2010) Differential Impact of Tetratricopeptide Repeat Proteins on the Steroid Hormone Receptors. PLoS ONE 5(7): e11717. doi:10.1371/journal.pone.0011717

**Editor:** Joanna Mary Bridger, Brunel University, United Kingdom

**Received:** November 25, 2009; **Accepted:** June 24, 2010; **Published:** July 22, 2010

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**Funding:** The research was financed by the Max Planck Institute of Psychiatry. Therefore, no funders had a role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

Steroid hormones are lipophilic signalling molecules, mediating a vast variety of physiological effects that depend on the cellular context of the target tissue. They act via steroid hormone receptors (SR), which belong to the nuclear receptor superfamily of ligand-activated transcription factors and serve as regulators of various target genes [1–3]. Upon binding to hormone, SR accumulate in the nucleus and either enhance or decrease transcription by interacting with their cognate DNA elements or by “cross-talk” with other transcription factors [4–6].

Hormone binding and activity of SR is shaped by molecular chaperones [7]. In general, molecular chaperones are highly conserved and abundant proteins that change the folding energy landscape for their client proteins to assist them in reaching their native conformation in an efficient and timely manner [8,9]. SR interact with a heterocomplex consisting of the heat shock protein (Hsp) 90, Hsp70, Hsp40, Hsp70/Hsp90 organizing protein (HOP),

p23 and various cochaperones in a stepwise fashion to attain a conformational state competent of binding to hormone with high affinity [10]. The model that emerged from research over the last two decades states that the initial folding steps are aided by Hsp70 based chaperones and co-chaperones, while the final steps are expedited through Hsp90-centred heterocomplexes [11].

Both Hsp70 and Hsp90 feature a C-terminal EEVD motif that serves as acceptor site for cochaperones that harbour a tetratricopeptide repeat (TPR) domain [12]. In particular the Hsp90-interacting TPR proteins have received broad attention as proposed regulators of SR function [11,13]. Among these TPR proteins are the carboxyl terminus of Hsc70-interacting protein (CHIP), Cyclophilin-40 (Cyp40), the immunophilin FK506-binding proteins (FKBP) 51 and 52, protein phosphatase 5 (PP5), the tetratricopeptide repeat protein 2 (TPR2) and the hepatitis virus B X-associated protein 2 (XAP2).

Many of the TPR proteins bring additional molecular functions to the SR-chaperone heterocomplexes. CHIP contains a C-

terminal U-box that interacts with ubiquitin-conjugating enzymes and has been reported to promote degradation of various steroid receptors [14–17]. The immunophilin and peptidylprolyl isomerase (PPIase) Cyp40 has also been identified in SR-heterocomplexes, but its role regarding SR-function is still unclear [18]. FKBP51, another PPIase, was characterised as a cellular factor contributing to the glucocorticoid resistance observed in New World primates [19,20]. It inhibits glucocorticoid receptor (GR) activity by lowering hormone binding affinity of the receptor and delaying nuclear translocation [20,21], and also inhibits the mineralocorticoid receptor [22]. In contrast, the highly homologous FKBP52 was found to positively modulate SR function and to be critical for progesterone and androgen function *in vivo* [18,23–27]. While the PPIase protein domains of FKBP51 and FKBP52 play an important role in GR's regulation, the function of the enzymatic PPIase activity remains enigmatic [18,21,28].

PP5 is the only TPR-domain containing phosphatase identified so far; it has been shown to modulate a variety of cellular pathways [29]. The role of PP5 in SR signalling appears controversial so far, possibly due to the different approaches and experimental systems. Both positive and negative modulatory effects of PP5 on steroid dependent transcription have been reported. Down-regulation of PP5 expression was shown to increase GR activity in reporter gene assays [30] and transcription of estrogen receptor (ER) target genes [31] suggesting a negative modulatory role of PP5 in steroid dependent signalling. In contrast, in a different study siRNA-mediated PP5 knock-down lead to a decrease in transcription of GR target genes [32]. In yeast, the PP5 homolog Ppt1 acts as a positive modulator of GR, possibly by removing inhibitory phosphates from Hsp90 [33].

TPR2 is a J-domain containing cochaperone which has been demonstrated to modulate GR and PR signalling [34,35]. It may act by mediating the retrograde transfer of substrates from Hsp90 onto Hsp70 [34]. XAP2 has been well studied for its role in regulating the activity of the arylhydrocarbon receptor (AhR) class of nuclear receptors [36]. Recently, XAP2 was shown to inhibit GR-mediated transcription [37].

Based on evidence from the literature and our own studies on the GR-inhibitory role of FKBP51, we had initiated a genotyping study that revealed a genetic association of this TPR protein with the response to medication in major depression [38]. Meanwhile, FKBP51 has been included in several studies and attracted great attention for the association of its genetic polymorphisms and gene expression level with a number of stress-related phenotypes and neuropsychological diseases, such as major depression or post traumatic stress disorder [38–46]. All these findings corroborate a physiological role of FKBP51 in stress regulation, most likely via its action in GR signalling.

Since several TPR proteins should be able to compete with FKBP51 for binding to the binding site for TPR proteins in an Hsp90 dimer [47–49], we assume that the overall impact of FKBP51, or any other TPR protein, on GR, or SR in general, depends on the relative abundance and mode of action of the other TPR proteins present in the same cell. Knowledge about each of these factors' capability to influence SR function might provide the basis for the understanding of tissue responsiveness to steroid hormones. A comprehensive comparison of the TPR-proteins on the function of all steroid receptors in a homogenous mammalian system has not been accomplished yet. Thus, we assessed the impact of the TPR proteins CHIP, Cyp40, FKBP51, FKBP52, PP5, TPR2 and XAP on each of the SR.

## Results

### Different responsiveness of the steroid hormone receptors in reporter gene assay

To set up an assay for the determination of the influence of the seven selected TPR proteins CHIP, CYP40, FKBP51, FKBP52, PP5, TPR2 and XAP2 on the six steroid receptors GR, MR, PR, AR, ER $\alpha$  and ER $\beta$ , we established reporter gene assays for each of the receptors. For GR, MR, PR and AR, we made use of the hormone-responsive elements of the MMTV LTR promoter that was linked to the structural part of the firefly luciferase gene [50]. To measure the activity of the two ER receptors we used a luciferase reporter plasmid with two copies of an estrogen responsive element instead of the MMTV LTR [51]. For each receptor, we used two sub-saturating concentrations of hormone as well as one concentration well in the range of saturation.

GR and PR displayed the widest, AR a considerable, and MR and the two ERs a moderate range of hormone inducible activity in human neuronal SK-N-MC cells (Fig. 1). We chose this cell line for two reasons, first because is largely devoid of steroid receptors, and second because of its neuronal origin.

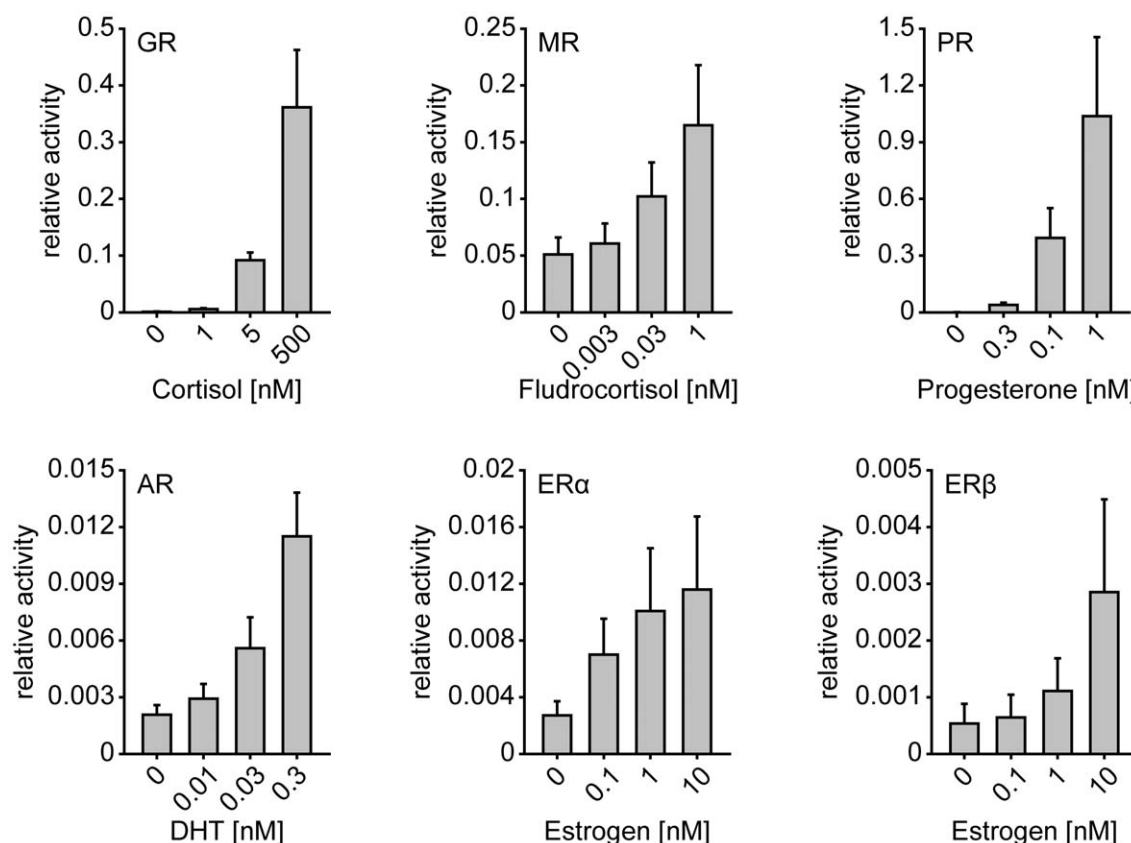
Since the effects of FKBP51 on GR have been reported to be most pronounced at sub-saturating concentrations of hormone, we focused our further analyses on conditions that yielded significant, but not yet full activation of the respective steroid receptor. In addition, we also included one saturating concentration of hormone for each receptor.

### Steroid receptors display differential sensitivity to TPR-proteins

To assess the effect of the TPR proteins on steroid receptor activity, each of the FLAG-tagged TPR proteins was co-expressed with each of the HA-tagged steroid receptors GR, MR, PR, AR, ER $\alpha$ , or ER $\beta$ , respectively, along with reporter and control plasmids. Since mammalian cells, in contrast to yeast, feature a number of different receptor-relevant TPR proteins, we reasoned that overexpression of a specific TPR protein is necessary to significantly enhance occupancy of the TPR acceptor site on Hsp90 by this specific cofactor. To test whether this is indeed the case under the conditions chosen we first evaluated the degree of overexpression for each of the TPR cofactors (Fig. 2A). Cells were transfected with plasmids encoding for one of the TPR proteins and probed their abundance in cell lysates using Western blot analysis. Each of the TPR cofactors was at least 4 fold enhanced over the endogenous levels (Fig. 2A).

Since our experimental design was further based on the assumption that selectively enhancing the level of one of the TPR cofactor results in changing the composition of the Hsp90 heterocomplexes, we tested this at the example of FKBP52 overexpression. Cells were transfected with FKBP52 expressing plasmid, and Hsp90 complexes were immunoprecipitated from cell lysates of FKBP52 overexpressing cells and control cells. While more FKBP52 was co-precipitated with Hsp90 complexes, all the other investigated TPR cofactors were less abundant (Fig. 2B, Cyp40 was below detection limit). As an important control, the interaction of Hsp90 with the non TPR protein p23 was not changed by increasing FKBP52 (Fig. 2B).

The first observation we made in the reporter gene assays was that, in general, the changes in receptor activity upon co-expression of TPR cofactors were more pronounced for GR, MR, and PR than for AR, and even more than for the two ERs, which were almost not affected (Figs. 3 and 4). Strong inhibitors of GR were CHIP, FKBP51, PP5, TPR2, and XAP2, while CYP40 and FKBP52 showed virtually no effect (Fig. 3A). As shown for



**Figure 1. Response of steroid hormone receptors in MMTV-reporter gene assays.** Neuronal SK-N-MC cells were transfected with a plasmid expressing one of the HA-tagged SRs, the MMTV firefly-luciferase reporter plasmid when transfecting GR, MR, PR, or AR, an ERE firefly-luciferase reporter plasmid for ER $\alpha$  and ER $\beta$ , and the Gaussia-KDEL control plasmid. After transfection, the cells were cultivated for 24 h in the presence of the indicated concentrations of hormone (DHT: Dihydrotestosterone) or EtOH as solvent control. Receptor activity represents firefly data normalized to Gaussia activities + S.E.M. of at least four independent experiments, each performed in duplicate.  
doi:10.1371/journal.pone.0011717.g001

GR, at saturating concentrations of hormone the inhibitory effect was greatly diminished, even though TPR2, for example, still reduced GR's activity twofold (Fig. 3B). Similar observations were made with saturating concentrations of hormone at the other receptors (data not shown).

The TPR reactivity profile of MR was very similar, except for PP5 and XAP, which exerted only a marginally inhibitory effect on MR (Fig. 3C). Albeit we were using stripped serum free of steroids, we observed a significant activity of MR even in the absence of added hormone, which was affected by the TPR cofactors in the same way as the hormone-stimulated activity (Fig. 3C and data not shown). To test whether any serum component might have contributed to hormone-independent activation of MR we cultivated transfected cells in serum free media for 24 h before measuring reporter activity. Serum withdrawal reduced MR-dependent transactivation, suggesting that factors other than glucocorticoids are present in steroid free media which partially activate MR's transcriptional activity (Fig. 3D). This effect appeared to be additive to the glucocorticoid-mediated effect, because stimulation with sub-saturating concentrations of fludrocortisol (0.03 nM) in stripped serum containing media resulted in higher MR activation than in serum free media (Fig. 3D).

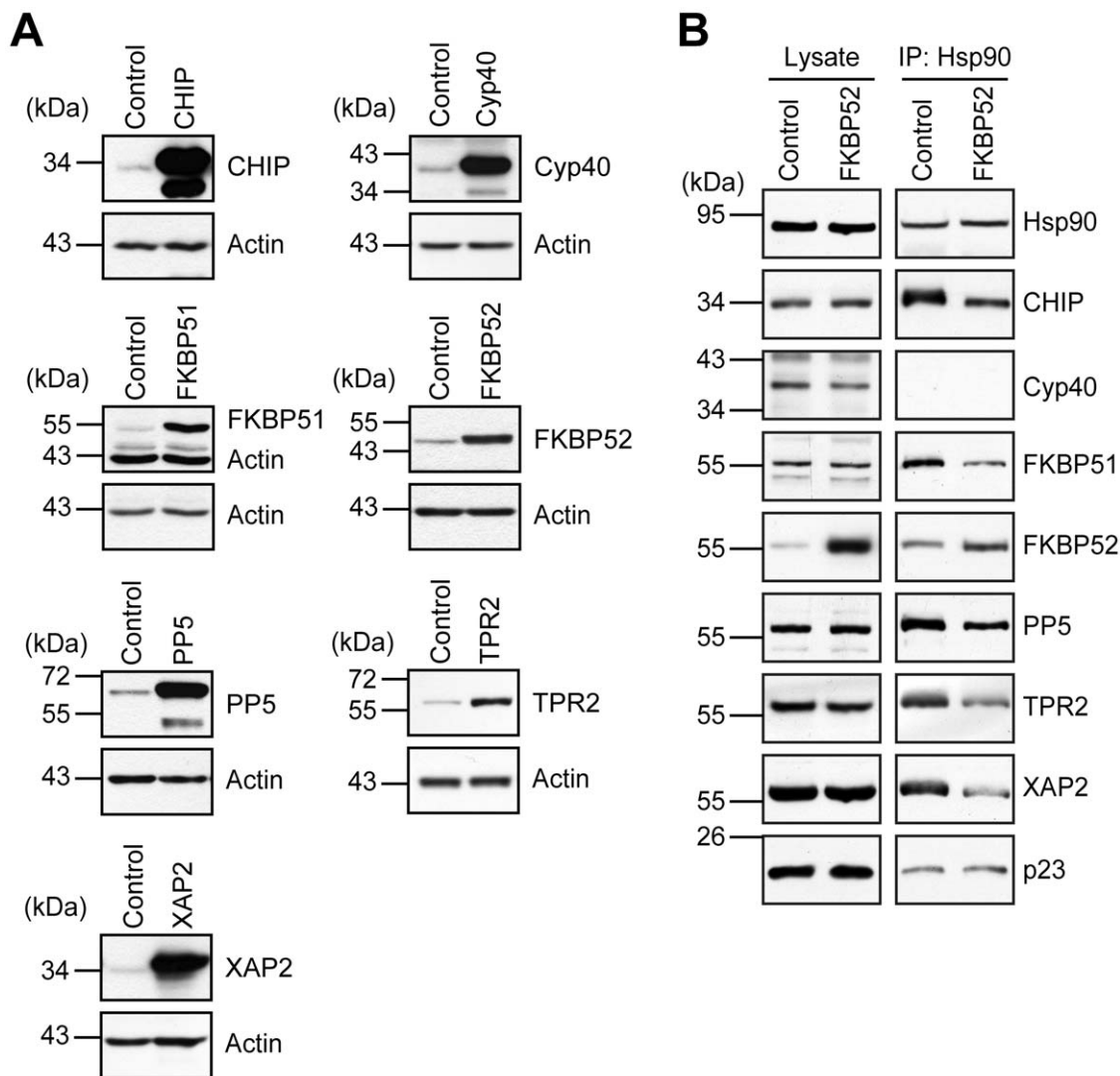
Similarly to GR, PR showed the highest activity when co-expressed together with CYP40 or FKBP52 (Fig. 4A). The effects of the TPR-cofactors were noticeably attenuated in the case of AR

(Fig. 4B). Only co-expression of FKBP52 maintained AR activity, while all the other TPR cofactors reduced this receptor's activity to a moderate degree, with TPR2 being the strongest inhibitor (5 fold inhibition, Figure 4B). ER $\alpha$  and ER $\beta$  showed almost no reaction to the presence of TPR cofactors under our conditions, except for TPR2, which reduced the activity of these receptors about 2 fold (Figs. 4C, D).

We also monitored the expression levels of the co-expressed receptors and TPR proteins. There were some variations throughout the experiments, but overall there were no gross alterations in the levels of the steroid receptors in dependence of the co-expressed TPR cofactor, except for CHIP which often, albeit not consistently, led to lower receptor expression levels (Figs. 3 and 4 examples in the panels below the activity assay graphs). This was not unexpected, because CHIP has been identified as E3 ligase and been shown to reduce the levels of GR [15]. The levels of the co-expressed TPR proteins also varied between experiments. Overall, Cyp40 and TPR2 had a tendency to be expressed at lower levels, and to a lesser extent also FKBP51, while the other cofactors expressed at the same levels.

### The estrogen receptors show little sensitivity to geldanamycin

Since most of the TPR proteins had little impact on ERs' transcriptional activity, we wondered whether these two receptors are dependent on functional Hsp90 at all under our assay



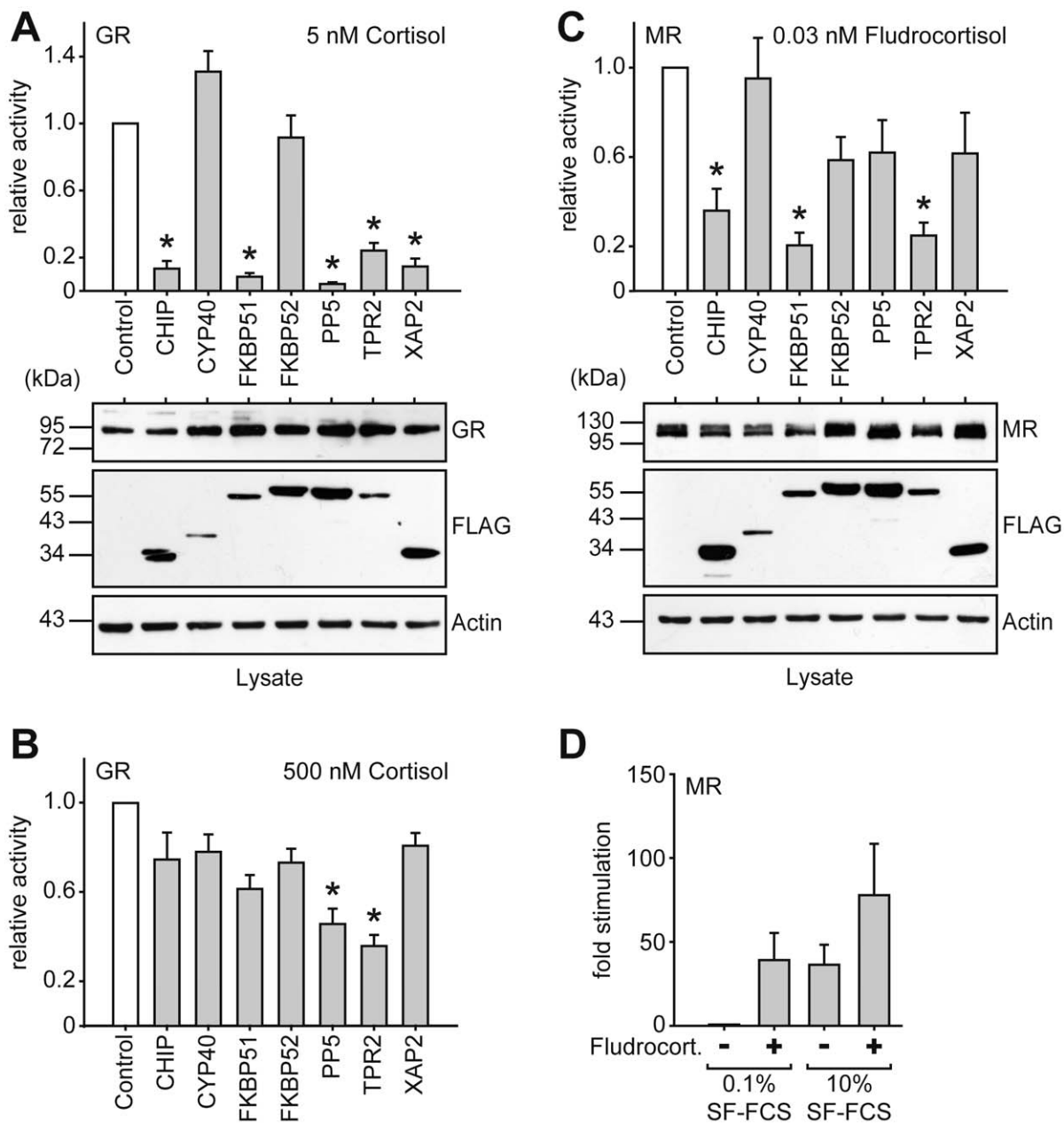
**Figure 2. TPR proteins are significantly enhanced upon ectopic expression and change Hsp90 heterocomplex composition.** A, SK-N-MC cells were transfected with plasmid expressing one of the TPR proteins, lysed after 48 h and levels of the respective TPR protein was determined by Western blot analysis. B, HEK-293 cells were transfected with FLAG tagged Hsp90 along with FKBP52 expressing plasmid or control plasmid. Hsp90 was precipitated from lysates and the levels of co-precipitated cofactors were determined by Western blot.  
doi:10.1371/journal.pone.0011717.g002

conditions. Therefore, we applied the specific Hsp90 inhibitor geldanamycin (GA), which has been shown to block Hsp90 activity by binding to its ATP pocket [52,53]. We performed reporter gene assays for ER $\alpha$ , ER $\beta$ , and GR in the presence or absence of GA (Fig. 5). While GA efficiently reduced the activity of GR (Fig. 4C), it had very little effect, if any, on the activity of ER $\alpha$  and ER $\beta$  (Figs. 5A,B). Whatever the reason for the apparent Hsp90-independent action of these two receptors is, it could explain why the Hsp90 cofactors have so little impact on their activity.

It has been found that GA leads to degradation of Hsp90 client proteins such as GR [54–56]. To test whether the differential responsiveness of GR and ER to GA is also reflected on the level of protein stability, we measured the expression levels of GR and ER upon GA treatment. Neither GR nor ER were significantly changed in their protein levels after exposure of transfected HEK cells (Fig. 5) or SK-N-MC cells (data not shown) with GA, which could be due to the much lower concentrations of GA used here compared to other studies [54,55].

### Cyclophilin 40 is unable to rescue receptor activity

FKBP52, which does not change the activity of GR when co-expressed with this receptor in mammalian cells (Fig. 3A, and [21]), has been shown to be able to attenuate the inhibitory effect of FKBP51 [21]. In our screen of the activity profiles of the TPR proteins Cyp40, like FKBP52, had no or very little effect on steroid receptors (Figs. 3 and 4). Thus, the question arose, whether an effect of Cyp40 on GR or MR may become apparent under conditions of compromised receptor activity, i.e. when an inhibitory protein is co-expressed. Therefore, we coexpressed FKBP51 at moderate levels to inhibit GR and MR, and added increasing amounts of Cyp40 expressing plasmid (Fig. 6). Even though Cyp40 was expressed up to levels that exceeded those of FKBP51 (both proteins were FLAG tagged, allowing a direct comparison in a Western blot) and well above endogenous levels, it was unable to rescue the activity of GR or MR (Figs. 6A and B). This is in contrast to FKBP52, which has been shown to be able to revert the inhibitory action of FKBP51 [21].

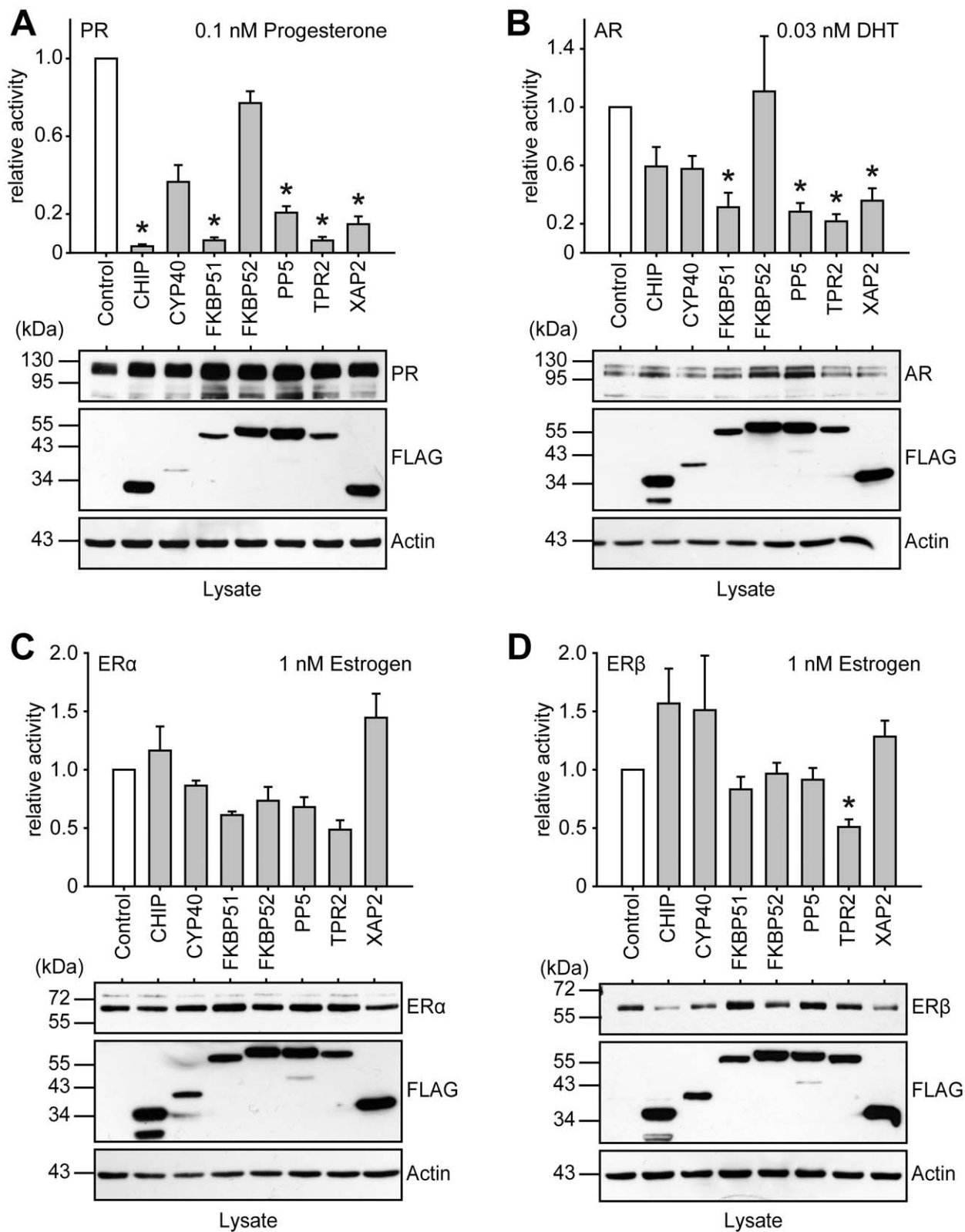


**Figure 3. GR and MR activities in the presence of different TPR-proteins.** A-C, SK-N-MC cells in 96 well plates were transfected with the MMTV-Luc, Gaussia-KDEL control plasmid, a plasmid expressing one of the HA-tagged steroid hormone receptor (GR in A and B, MR in C and D) and constant amounts (200 ng) of a plasmid expressing one of the FLAG-tagged TPR-proteins. After transfection, the cells were cultivated for 24 h in the presence of hormone or vehicle as indicated. Relative receptor activity represents firefly data normalized to Gaussia activities and presented as relative stimulation to control + S.E.M. of at least four independent experiments performed in duplicate. Control cells were transfected with cloning plasmid instead of the TPR protein expressing plasmid. Lower panels of A and C, immunoblot of cell extracts, probed with anti-HA antibody visualizing steroid receptor expression, the same membrane probed with FLAG antibody demonstrating expression of TPR proteins and with actin antibody as loading control. D, After transfection, cells were cultivated in 0.1% or 10% SF-FCS containing media for 24 h in the presence of 0.03 nM fludrocortisol, or EtOH as vehicle control. Firefly luciferase data were normalized to Gaussia luciferase activities and are presented as relative stimulation + S.E.M. of three independent experiments performed in triplicate. \* denotes  $p$ -values  $\leq 0.001$ . doi:10.1371/journal.pone.0011717.g003

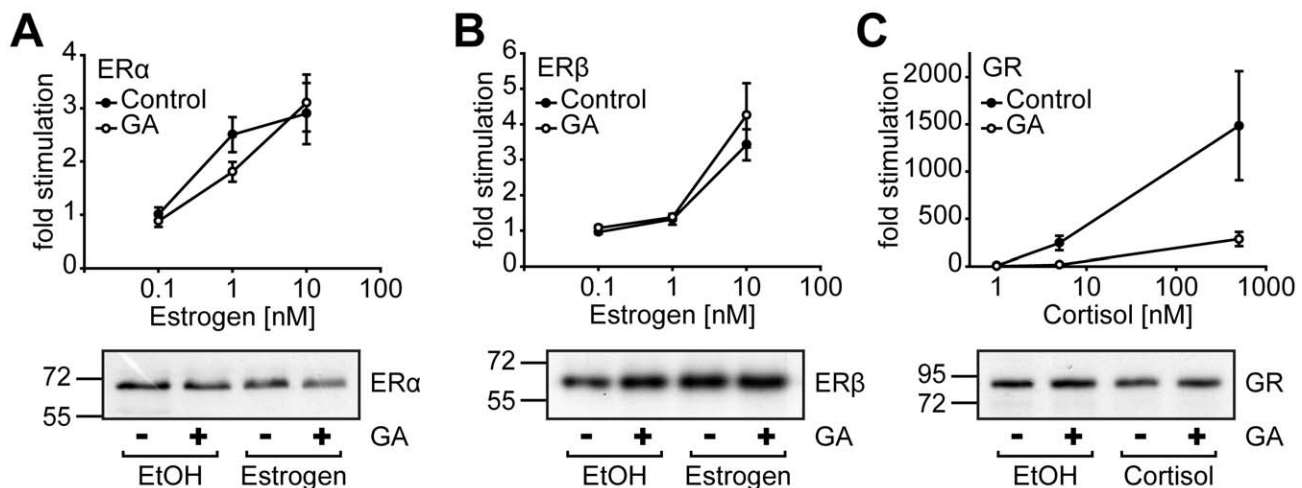
### Binding profiles of TPR proteins to steroid receptor-Hsp90 heterocomplexes

The ability of TPR proteins to access heterocomplexes of steroid receptors and Hsp90 is assumed as prerequisite for their impact on these receptors. Therefore, we evaluated the relative incorporation of the TPR-proteins into steroid receptor complexes employing complementary co-immunoprecipitation. The estrogen receptors

were not included, because they were only marginally affected by most of the TPR proteins. We expressed each of the HA-tagged steroid receptors in combination with each of the seven FLAG-tagged TPR proteins and performed co-immunoprecipitations with antibodies directed against the HA-tagged receptors or the FLAG-tagged TPR proteins, respectively, and visualized co-precipitated proteins by Westernblot analysis.



**Figure 4. PR, AR, ERα and ERβ activities in the presence of different TPR proteins.** SK-N-MC cells were transfected with the MMTV-Luc (for PR and AR assays), or the ERE-Luc reporter plasmid (for ERα and ERβ assays), the Gaussia-KDEL control plasmid, a plasmid expressing the HA-tagged steroid hormone receptor as indicated and the plasmid expressing a FLAG-tagged TPR-protein. After transfection, cells were cultivated for 24 h in the presence of hormone as indicated. Relative receptor activity represents firefly data normalized to Gaussia activities and presented as relative stimulation to control + S.E.M. of at least four independent experiments performed in duplicate. Control cells were transfected with cloning plasmid replacing the TPR protein expression plasmid in the transfection mixture. Lower panels of A–D display immunoblots of cell extracts, probed with anti-HA antibody visualizing steroid receptor expression, the same membrane probed with FLAG antibody demonstrating expression of the TPR proteins and with actin antibody as loading control. \* denotes  $p$ -values  $\leq 0.001$ . doi:10.1371/journal.pone.0011717.g004



**Figure 5. Estrogen receptors display little sensitivity to the Hsp90 inhibitor geldanamycin.** SK-N-MC cells were transfected with 0.25  $\mu$ g of one of the plasmids expressing ER $\alpha$  (A), ER $\beta$  (B) or GR (C), together with either ERE-Luc (A,B) or MMTV-Luc (C) as reporter plasmid and the Gaussia-KDEL control plasmid. After transfection, the cells were cultivated for 24 h in the presence of hormone and 10 ng/ml GA as indicated. Relative receptor activity represents Firefly data normalized to Gaussia activities and is presented as relative stimulation to control  $\pm$  S.E.M. of at least four independent experiments performed in duplicate. Lower panels, analysis of receptor expression after GA treatment in the presence or absence of hormone (10 nM estrogen, 500 nM cortisol). doi:10.1371/journal.pone.0011717.g005

Since we observed varying efficiencies in the amount of precipitated protein using HA- or FLAG-directed antibodies, co-precipitated proteins were normalized to the precipitated primary target, and in case of HA-directed IPs also to the relative expression of the different TPR proteins.

For GR, the receptor IP revealed CHIP, FKBP51 and TPR2 as strong binders to the heterocomplex (Fig. 7). The FLAG-IPs targeting the TPR proteins revealed a similar binding pattern. We observed that Cyp40 exhibited weak interaction with the heterocomplex, which may account for its inability to compete the inhibitory effect of FKBP51. Notably, the strongest binders all were strongly inhibitory proteins. On the other hand, PP5, which also significantly reduced GR activity, in comparison displayed only moderate interaction.

For MR, the interaction pattern of the TPR cofactors was similar to that of GR. Again, CHIP, FKBP51 and TPR2 exhibited strong interaction, while Cyp40 showed very little binding, both when immunoprecipitating the receptor or the cofactor (Fig. 8 A and B). Of note, the inability of PP5 to inhibit MR's transcriptional activity was not reflected by a corresponding low incorporation into MR heterocomplexes.

In the case of PR, we observed the strongest interaction with the PR-Hsp90 heterocomplex for CHIP, FKBP51, and TPR2 (Fig. 9), which in the reporter gene assay also were the ones that exhibited the strongest inhibitory activity (Fig. 4A). Remarkably, XAP2 and PP5, which also reduced PR's transcriptional activity, bound only weakly to the complex.

Although AR showed less activity change in response to co-expression of TPR cofactors than GR, MR and PR, the TPR cofactors exhibited a distinct binding profile (Fig. 10). The most efficient binding was observed for TPR2, in the presence of which AR was least transcriptionally active (Fig. 4B). In general though, there was no strict correlation between binding efficiency to the Hsp90-AR heterocomplex and the influence on the transcriptional activity of AR. For example, in the presence of CHIP or Cyp40, AR exhibited a very similar transcriptional activity, but these two TPR proteins differed markedly in their ability to access the AR-Hsp90 heterocomplex (Figs. 4B and 10).

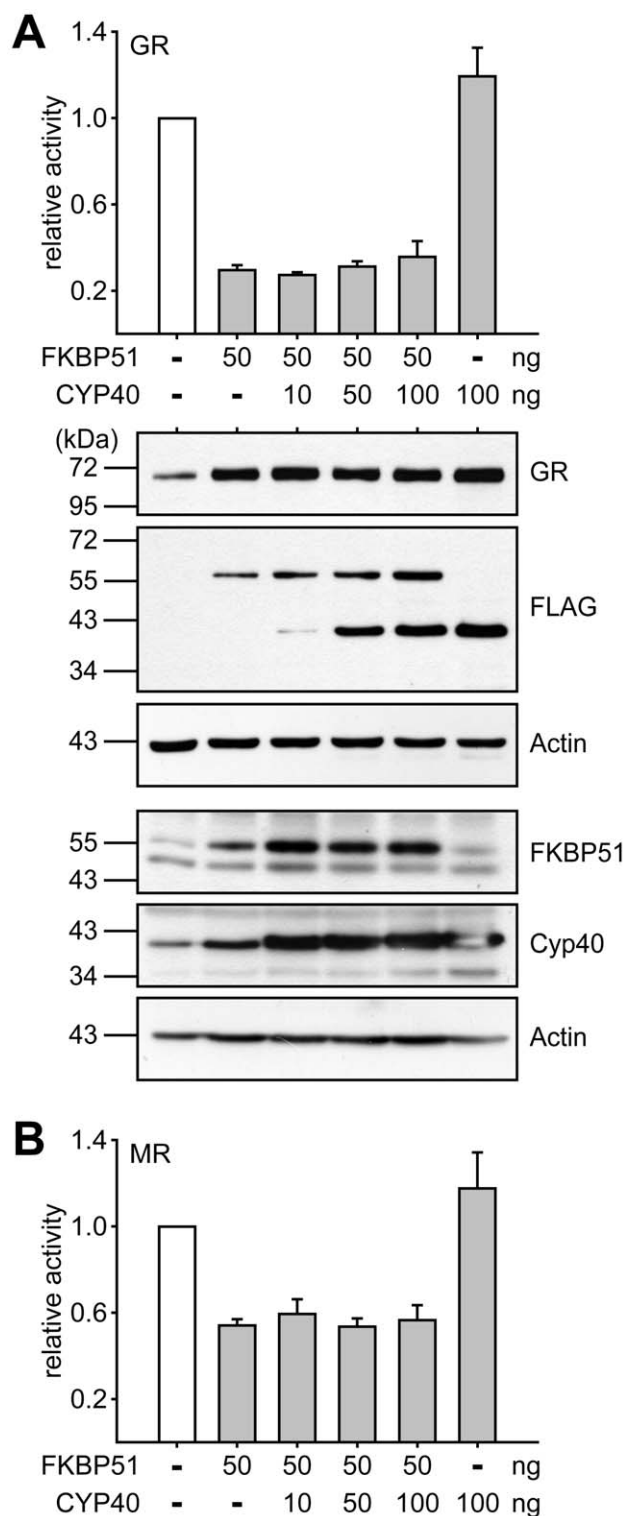
### TPR cofactors favor differently composed multi-chaperone heterocomplexes

During maturation, the steroid receptor proceeds through a multi-chaperone machinery in which each step is characterized by a relative abundance of distinct chaperones [7]. Therefore, it is possible that preference of the TPR cofactors to distinct heterochaperone complex compositions represents an important mechanistic aspect of their function. Thus, we compared the abundance of endogenous components of the chaperone machinery co-precipitating with the immunoadsorbed TPR-cofactors.

Since the FLAG-tagged proteins were precipitated with different efficiencies (although amounts of plasmids were adjusted so that the TPR proteins were expressed at similar levels, compare Figs. 7–10), the amount of co-precipitated Hsp90, Hsp70 and p23 was normalized to the amount of precipitated TPR cofactor. We consistently observed some nonspecific binding of Hsp70 to the FLAG agarose resin, and therefore, considered only levels exceeding the background binding as indicative of Hsp70 interaction. Co-expression of the different steroid receptors did not change the relative co-precipitation of Hsp70, Hsp90 and p23. Therefore, we used the results of experiments with different steroid receptors to determine the relative binding of these components to the TPR-proteins.

Hsp90 interaction was detected for all TPR cofactors investigated here, as expected. However, there was a considerable difference in the relative amount of co-precipitated Hsp90 (Fig. 11A and B). FKBP51 and FKBP52 displayed the strongest Hsp90 interaction, and PP5 still about 4 fold higher interaction than CHIP, CYP40, TPR2 and XAP2, which all bound at comparable levels. Notably, while p23 interaction reflected the relative Hsp90 co-precipitation in general, p23 co-precipitated with FKBP52 less than with FKBP51 or PP5 in relation to the Hsp90 association (Fig. 11C). Apparently, FKBP51 and PP5 favor p23 containing Hsp90 heterocomplexes more than FKBP52, possibly by stabilizing the interaction between Hsp90 and p23.

Hsp70 binding was detected for CHIP and TPR2, as reported previously [34,57]. No Hsp70 binding was detected for CYP40, FKBP52, PP5 and XAP2, but surprisingly, for FKBP51. Although



**Figure 6. Cyp 40 is unable to compete the inhibitory effect of FKBP51.** SK-N-MC cells were transfected with the MMTV-Luc reporter plasmid, the Gaussia-KDEL control plasmid, one of the plasmids expressing the HA-tagged GR or MR as indicated, and plasmids expressing FKBP51 and Cyp40 at the indicated amounts. After transfection, the cells were cultivated for 24 h in the presence of 10 nM cortisol (A) or 0.03 nM Fludrocortisol (B). Bar graphs indicate the relative reporter activity representing Firefly measurements normalized to Gaussia activities and presented as relative stimulation + S.E.M. of three independent experiments performed in triplicate. Lower panel of

A displays immunoblots of cell extracts, probed with HA antibody demonstrating GR expression and the same membrane probed with FLAG antibody to detect overexpressed FKBP51 and Cyp40, and actin as control. In addition, antibodies directed against FKBP51 or Cyp40 were used to visualize the combined levels of endogenous and ectopic TPR protein.

doi:10.1371/journal.pone.0011717.g006

this binding was clearly weaker than that observed for CHIP and TPR2, it was significantly more than the virtually non existent Hsp70 binding of FKBP52 ( $p = 0.007$  in an unpaired student's t-test). This could indicate that FKBP51 favors early stages of the folding cycle, which could contribute to its inhibitory function.

### Loss of FKBP52 impairs GR function

The experiments described so far were based on increasing the abundance of a specific TPR cofactor in Hsp90 heterocomplexes. Considering the plethora of TPR cofactors in the cell, we pondered on the ability of mammalian cells to compensate for the loss of one of the proteins. Based on the inability of enhanced FKBP52 to significantly increase GR function, we reasoned that loss of an inhibitory factor, for example FKBP51, would have little effect. Therefore, we experimentally addressed the effect of loss of the established positive GR regulator FKBP52. Since our attempts to reduce FKBP52 using si-RNA resulted in only partial reduction (data not shown), we used mouse embryonic fibroblast (MEF) FKBP52 KO and WT cells. We found that stimulation of GR activity at saturating concentrations of hormone was not significantly affected (data not shown). However, higher concentrations of hormone were needed in FKBP52 ko cells to elicit a GR response comparable to that in WT MEF cells (Fig. 12).

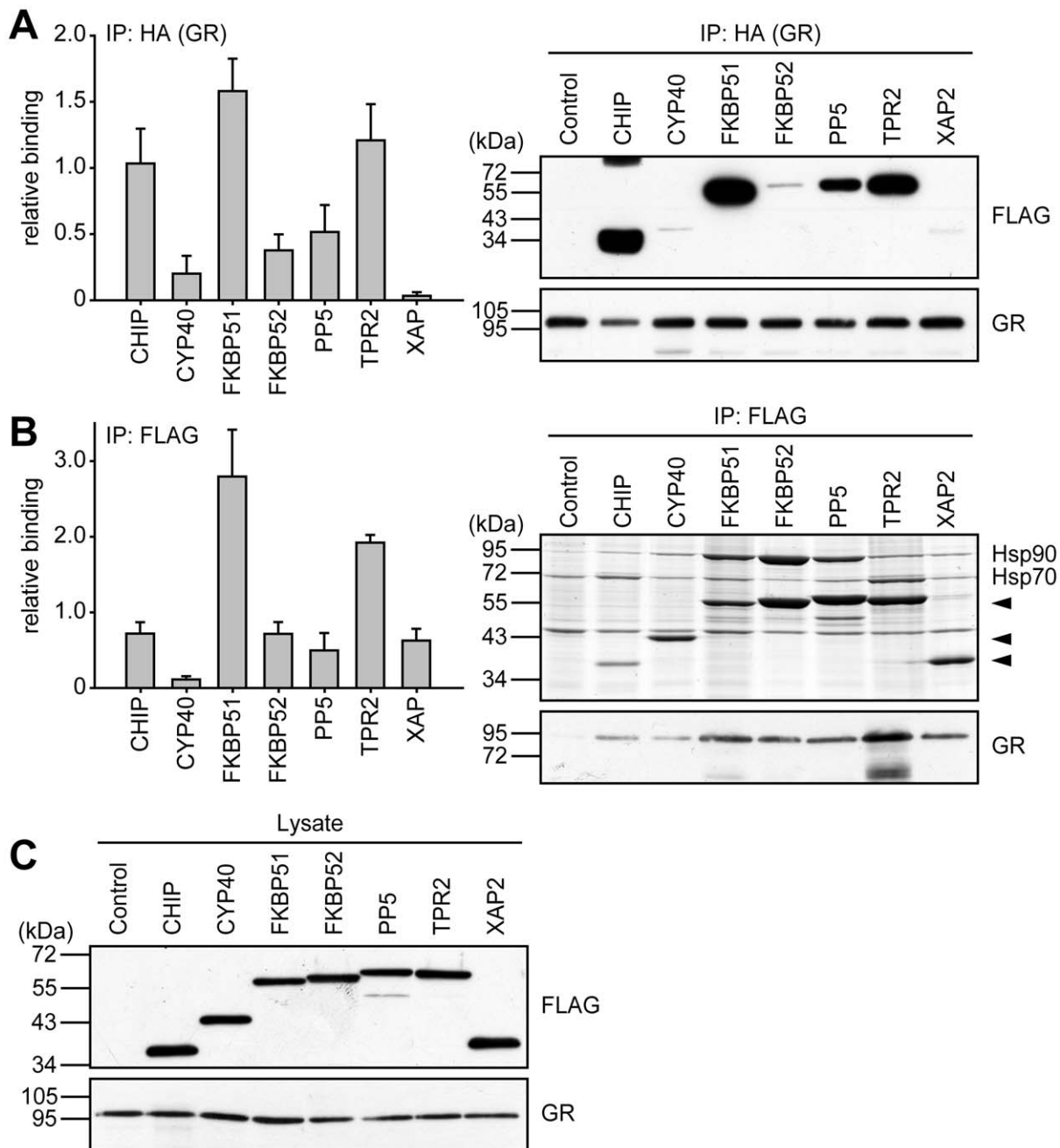
Since cells derived from different animals and cultivated for several generations can differ in numerous factors, it was mandatory to test whether the difference in the cortisol responsiveness between WT and FKBP52 KO MEF cells was indeed due to loss of FKBP52. Therefore, we overexpressed FKBP52 in FKBP52 KO MEF cells, which rendered the cortisol responsiveness indistinguishable from that of WT MEF cells (Fig. 12).

### Discussion

How are molecular chaperones able to assist correct folding of a plethora of structurally divergent proteins? In general, the various chaperone factors protect non-native protein chains from misfolding and aggregation, but do not contribute conformational information to the folding process [8]. They interact with features of non-native protein folds that are common to many proteins, such as hydrophobic stretches and unstructured backbone regions, and provide nano-compartments to shield proteins during their folding process from other proteins. Hsp90 regulates mainly a wide range of signal transduction molecules, and thus belongs to the more specialised, but still very versatile chaperones [9,58]. Our study provides a better understanding of this versatility through combinatorial compositions of the Hsp90-client heterocomplexes.

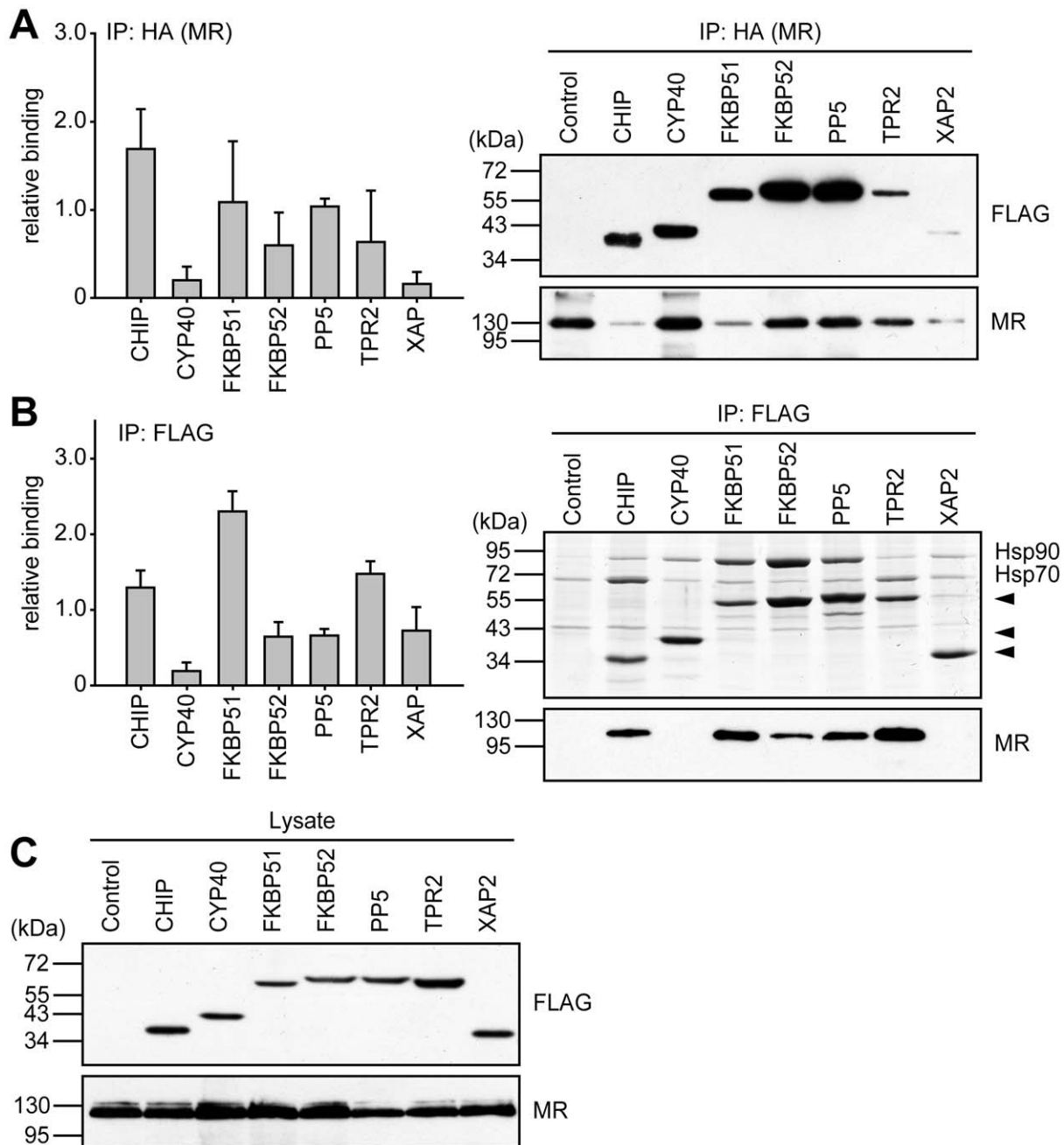
Of the six steroid receptors, the closely homologous GR, MR and PR exhibited the strongest reaction to changes in the TPR-protein make-up of the cell (Fig. 2 and 3). AR, and the ERs in particular, were less affected by co-expressing any of the co-chaperones. This may be explained by a diminished Hsp90-dependency of ER, at least in our cellular set-up, corroborated by the ineffectiveness of GA towards ER. Others have also provided evidence that ER may operate independently of Hsp90 [59,60], which contrasts reports on lower ER activity where Hsp90 function was compromised [61–64]. It should be noted, though,





**Figure 7. TPR-proteins differently interact with GR heterocomplexes.** HEK-293 cells were transfected with 5  $\mu$ g of a plasmid expressing HA-tagged GR together with 2–10  $\mu$ g (to achieve similar expression levels) of one of the plasmids expressing a FLAG-tagged TPR protein. After 48–72 h cultivation in SF-FCS containing media, cells were harvested, lysed, and protein extracts prepared for immunoprecipitation of either the HA-tagged GR (A), or the FLAG-tagged TPR-proteins (B). A, Precipitation of HA-GR. Displayed is an example of an immunoblot that was probed with FLAG antibody to visualize co-precipitated TPR-proteins (upper right panel), and an immunoblot of the same membrane probed with HA antibody demonstrating precipitated GR (lower right panel). Left panel, quantification of the relative binding of the TPR-proteins to the steroid receptor heterocomplexes. FLAG- and HA-immunoblot signals of the eluates and FLAG immunoblot signals of the cell extracts, demonstrating expression of TPR proteins (C), were scanned and subjected to densitometry. The signal from the co-precipitated FLAG protein was corrected first by the amount of precipitated receptor and second by the amount of the TPR-protein present in the respective cell extract. Binding of TPR-proteins is presented relative to the mean of the normalized FLAG-eluate signals of CHIP, FKBP51, FKBP52, and PP5. Quantification represents the means of three independent experiments  $\pm$  S.E.M. B, precipitation of TPR proteins. Upper right panel, coomassie stained gel of eluates visualizing precipitated TPR-proteins (arrowheads) and co-precipitated Hsp90 and Hsp70. Lower right panel, immunoblots of eluates probed with HA antibody to demonstrate binding of GR to TPR-protein heterocomplexes. Left panel, quantification of the relative binding of co-precipitated proteins to the precipitated TPR-proteins. For quantification, signals were scanned and subjected to densitometry. Each HA immunoblot signal of the eluate was corrected by the amount of precipitated TPR-protein. Binding of steroid receptors is presented relative to the mean of the corrected HA eluate signals. Quantifications represent means of three independent experiments  $\pm$  S.E.M.

doi:10.1371/journal.pone.0011717.g007



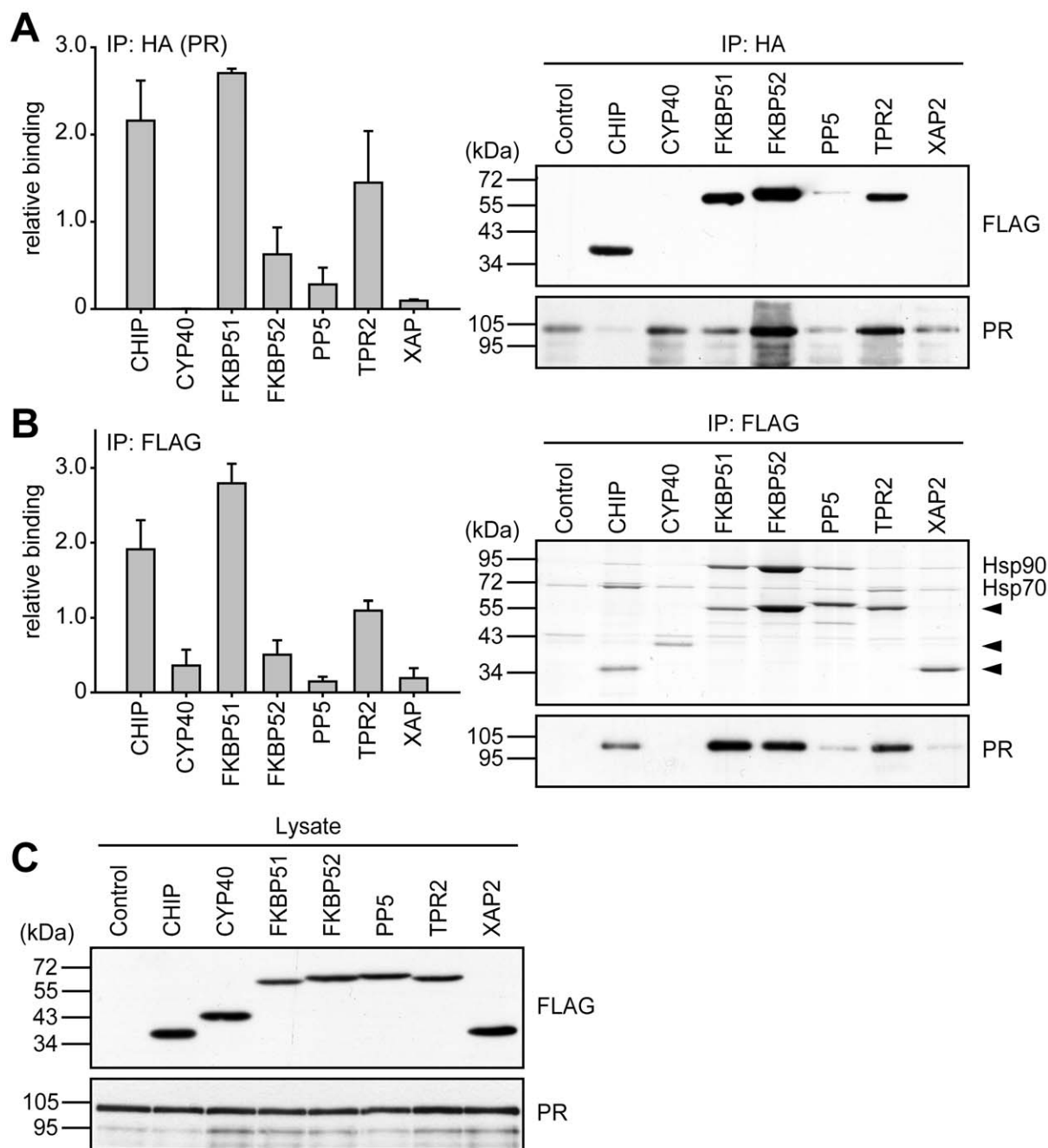
**Figure 8. Differential interaction of TPR-proteins with MR heterocomplexes.** HEK-293 cells were transfected as described for figure 7, except that HA-MR was expressed instead of HA-GR. Cells were processed and protein interactions were analyzed also as described for figure 7. In A, binding of TPR-proteins is presented relative to the mean of the normalized FLAG-eluate signals of CHIP, FKBP51, FKBP52 and PP5. Quantification represents means of three independent experiments (two for TPR2) +S.E.M.. In B, binding is normalized as in figure 7. C, FLAG- and HA-immunoblot signals of the cell extracts, demonstrating expression of TPR proteins and MR. Quantifications represent means of three independent experiments +S.E.M.

doi:10.1371/journal.pone.0011717.g008

that high doses of GA of 0.2–1  $\mu\text{g/ml}$  have been used in these reports. We used a 20–100 fold lower concentration of GA, which efficiently reduced GR activity, like we also have observed previously [65]. We cannot exclude the possibility that ER activity could be impaired also in our cellular system at very high concentrations of GA, which however, would raise the question of non-specific effects of GA. We propose that the Hsp90-dependency of ER is cell-type dependent, and possibly affected

by the presence or absence of additional, yet to be revealed factors. In addition, high doses of GA have been reported to induce reactive oxygen species in cells [66–69], which might contribute to differences in the effects of GA on ER at different concentrations.

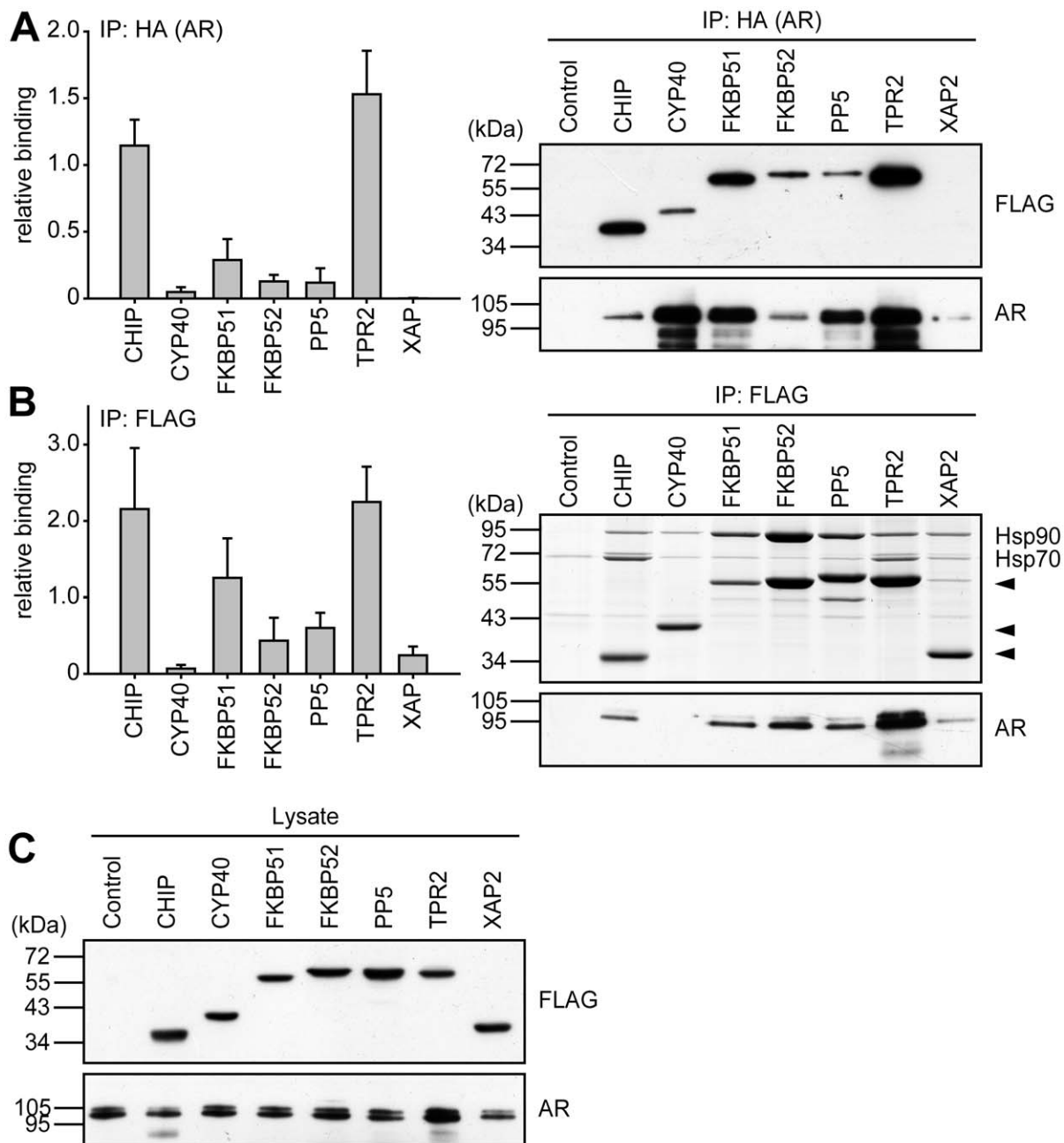
Our study also documents numerous differences in the efficacies of the TPR proteins' influence on SR. Cyp40 exhibited only a minor effect on AR and PR, and no effect on GR, MR and the ERs, which concurs with its small binding affinity to Hsp90 and



**Figure 9. Differential interaction of TPR-proteins with PR heterocomplexes.** HEK-293 cells were transfected as described for figure 7, except that HA-MR was expressed instead of HA-GR. Cells were processed and protein interactions were analyzed also as described for figure 7. In A, binding of TPR-proteins is presented relative to the mean of the normalized FLAG-eluate signals of CHIP, FKBP52, PP5 and TPR2. Quantification represents means of three independent experiments (two for FKBP51) +S.E.M. In B, binding is normalized as in figure 7. C, FLAG- and HA-immunoblot signals of the cell extracts, demonstrating expression of TPR proteins and PR. Quantifications represent means of three independent experiments +S.E.M.  
doi:10.1371/journal.pone.0011717.g009

steroid receptor heterocomplexes in comparison to other TPR proteins (Fig. 6–10). Work in yeast, which expresses the two Cyp40 homologues Cpr6 and Cpr7, revealed an involvement of Cpr7, but not Cpr6 in the hormone-dependent activity of GR [70,71]. In addition, Cpr6 did not influence Hsp90 activity [72]. In mammalian cells, the effect of Cyp40 on steroid receptors has not been directly assessed. However, cyclosporine A, which is known to target Cyp40 as well as Cyp18, somewhat diminished AR function in LNCaP cells [73].

CHIP efficiently inhibited the transactivational activity of GR, MR, PR, and moderately affected AR. It has been reported that CHIP induces degradation of GR, AR and ER $\alpha$  [15–17] and reduces hormone binding of GR [15]. With respect to steroid receptor degradation, we observed a tendency towards lower receptor amounts, but no consistently significant effect. Since saturating concentrations of hormone greatly attenuated the inhibitory effect of CHIP on all steroid receptors (Fig. 2B for GR and data not shown for MR, PR, AR), mechanisms in

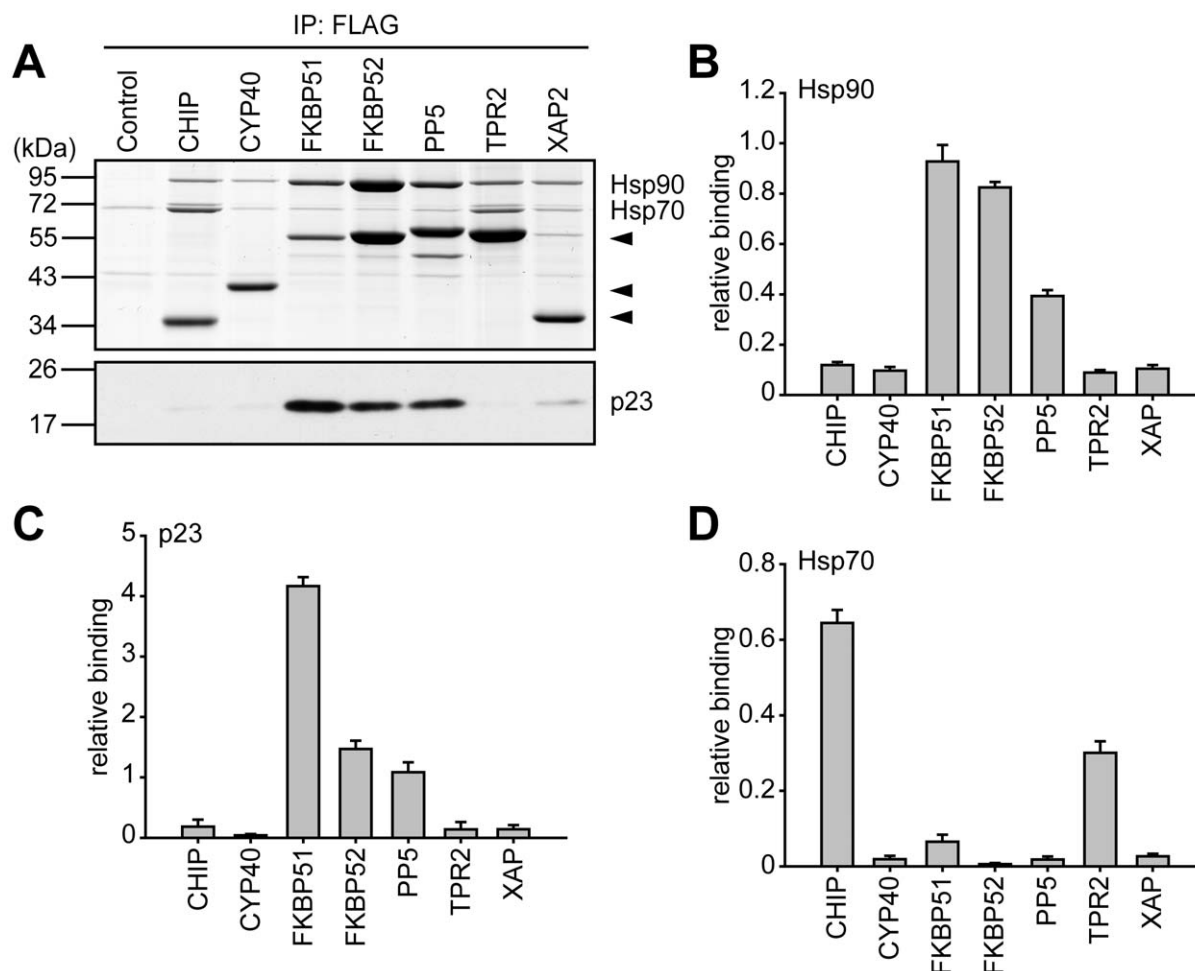


**Figure 10. Differential interaction of TPR-proteins with AR heterocomplexes.** HEK-293 cells were transfected as described for figure 7, except that HA-MR was expressed instead of HA-GR. Cells were processed and protein interactions were analyzed also as described for figure 7. In A, binding of TPR-proteins is presented relative to the mean of the normalized FLAG-eluate signals of CHIP, FKBP51, and TPR2. Quantification represents means of three independent experiments (two for XAP2) +S.E.M. In B, binding is normalized as in figure 7. C, FLAG- and HA-immunoblot signals of the cell extracts, demonstrating expression of TPR proteins and AR. Quantifications represent means of three independent experiments +S.E.M.  
doi:10.1371/journal.pone.0011717.g010

addition to protein degradation must be responsible for the observed inhibition, most likely reduction of hormone binding. Moreover, the interaction of CHIP with Hsp70 may lead to an influence on SR at early stages of the folding cycle, similarly to TPR2 [34]. AR may be a special case, as CHIP interacts not only via Hsp90 with the LBD of this receptor, but also via its C-terminus with a conserved motif at the N-terminus of the receptor [17].

Increasing or reducing the levels of TPR2 has been shown to reduce the activity of GR and PR [34,35], while other steroid

receptors had not been analyzed before. In our experiments, increased levels of TPR2 resulted in a strong reduction of the activity of all SR, in contrast to the other investigated TPR proteins, which exhibited at least some selectivity in their action on SR. Our finding of strong interaction of TPR2 with Hsp70, but only moderate interaction with Hsp90 in comparison with other TPR proteins, supports the hypothesis that TPR2 acts by interference at early stages of the SR folding cycle [34,35]. Furthermore, TPR2 still displayed considerable inhibitory activity at saturating conditions of hormone. Thus, TPR2 most likely



**Figure 11. TPR cofactors differentially recruit components of the multichaperone heterocomplex.** HEK cells were transfected and TPR cofactors immunoprecipitated as described in the legends to figures 7–10. The relative amounts of the precipitated TPR cofactors, the co-precipitated Hsp70 and Hsp90 were determined by densitometry of a coomassie stained gel of the eluates (A, upper panel), and the relative amount of p23 by densitometry of the immunoblot signals (A, lower panel). B and D, quantification of the relative binding of co-precipitated Hsp90 (B) and Hsp70 (D). Hsp90 signals and Hsp70 signals (only intensities above background binding were taken into consideration) were normalized to signals of the respective precipitated TPR cofactors. Data are presented as relative binding + S.E.M. of at least 12 independent experiments with different steroid receptors. C, quantification of the relative binding of co-precipitated p23. The p23 immunoblot signals were related (normalized) to the respective TPR cofactor signal. Binding of p23 is presented relative to the mean of the normalized p23 eluate signals of the complete set of TPR-proteins. doi:10.1371/journal.pone.0011717.g011

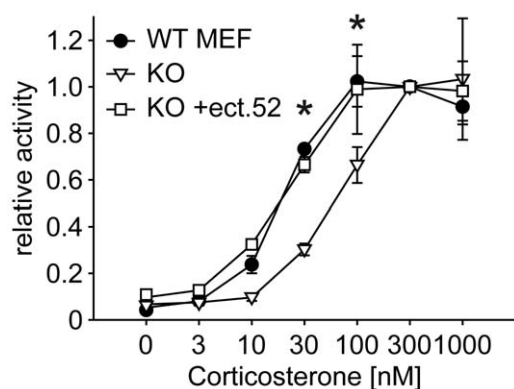
operates through mechanisms in addition to reducing hormone binding affinity [34].

For XAP2, a moderate interaction with Hsp90 has been found before [37,74], but there were no reports on incorporation into SR heterocomplexes. We reveal here the potential of XAP2 to interact with SR. This leads to a differential impact on the transcriptional activity of the receptors, with the strongest effects observed for GR and PR, while MR displayed little reaction to the presence of XAP2. XAP2 also interacts with other receptors, such as AhR [74], peroxisome proliferator-activated receptor  $\alpha$  [75] and thyroid hormone receptor  $\beta$ 1 [76]. These interactions go along with an inhibition of the transcriptional activity of PPAR $\alpha$ , and a stimulation of AhR and THR $\beta$ 1. XAP2 also affects nuclear translocation of AhR [77,78] and GR [37].

FKBP51 and FKBP52 are the most intensely investigated TPR cofactors of steroid receptors. In particular for GR, important insight was gained from experiments in yeast, that characterised FKBP52 as stimulatory GR cofactor, while FKBP51 had no effect [18]. Studies in mammalian cells reported a strong inhibitory

action of FKBP51 on GR, while over-expression of FKBP52 had no effect [20,21,79,80]. In at least some mammalian cells, a positive effect of FKBP52 on AR- and GR-signaling has been observed [23,24,81]. Gene knock-out studies in mice revealed an essential influence of FKBP52 on AR- and PR-related physiological processes, while ablation of the FKBP51 gene did not result in an overt phenotype [24–27]. Very recently, a stimulatory effect of FKBP51 on AR has been reported in prostate cancer cells [82,83]. We have obtained preliminary evidence that this may be a cell-type-specific effect (data not shown).

In the study presented here, FKBP51 and FKBP52 exhibited divergent effects on the transcriptional activities of GR, MR, PR and AR. Consistent with a previous report on GR [84], we also observed a stronger incorporation of FKBP51 in SR heterocomplexes than of FKBP52. At the same time, the interaction of both proteins with Hsp90 was comparable. Thus, the interaction with Hsp90 is not the sole determinant for the efficiency of integration into SR-heterocomplexes. Our interaction analyses further revealed a higher abundance of p23 in Hsp90 complexes



**Figure 12. Loss of FKBP52 affects GR responsiveness to cortisol.** FKBP52-KO MEF cells (open symbols) or WT MEF cells (closed circles) were transfected with the MMTV-Luc reporter plasmid, the Gaussia-KDEL control plasmid, a plasmid expressing the HA-tagged mGR and either a plasmid expressing FLAG-tagged FKBP52 (+ect.52) or empty vector. After transfection, cells were cultivated for 24 h in the presence of hormone. Relative receptor activity represents firefly data normalized to Gaussia activities and is presented relative to the activity at saturating 300 nM corticosterone  $\pm$  S.E.M. of three independent experiments, each performed in triplicates. Significance of different receptor activation between FKBP52 KO cells and FKBP52 KO cells ectopically expressing FLAG-tagged FKBP52 was evaluated by one sampled T-test (\* denotes  $p$ -values  $\leq 0.001$ ). doi:10.1371/journal.pone.0011717.g012

with FKBP51 than in Hsp90 complexes with FKBP52. This may be explained by the possibly different interaction surfaces of Hsp90 that are engaged in binding these two immunophilins. FKBP52, in addition to the classic C-terminal MEEVD motif, recognises amino acids at the ATP binding pocket [85], which may impinge on p23 interaction. It should be noted, though, that binding of FKBP51 to this N-terminal site has not been tested yet [85]. Whatever the explanation is for the differential recruitment of p23, the increased presence of p23 may be related to the inhibitory action of FKBP51 on GR [86]. In addition, the recruitment of Hsp70 by FKBP51, albeit minor, could also add to the differential action of FKBP51 and FKBP52, as Hsp70 is absent in mature receptor heterocomplexes.

Our experiments revealed an inhibitory effect of PP5 that was most pronounced in the case of GR. Previous studies examining the effect of PP5 on GR produced partly inconsistent results. Expression of the TPR domain of PP5 in CV-1 cells abolished GR-dependent transcription [87], like probably the over-expression of any functional TPR-domain would do. On the other hand, over-expression of the PP5 TPR domain slightly stimulated the transactivation of ER $\alpha$  and ER $\beta$  [31], which reinforces the notion that the ERs differ in their TPR-protein dependency from the other SRs. Expression of full-length PP5 inhibited ER $\alpha$  and ER $\beta$ , probably via dephosphorylation of ER [31]. Knock-down of PP5 increased GR-dependent reporter gene activity in one study [30], while another study discovered a reduction of endogenous mRNA levels of GR-dependent genes [32]. In yeast, no effect of PP5 on GR was observed and PP5 was unable to compete with FKBP52 to decrease the GR-stimulation of this protein [18]. The inactivity of PP5 in yeast, which is insufficiently endowed with TPR-cofactors positive for GR, is compatible with our observations of the inhibitory action of PP5 in mammalian cells. Another study in yeast reported a positive effect of the PP5 yeast homologue Ppt1 on GR function, possibly due to removal of chaperone-inhibitory phosphates on Hsp90 [33]. It is not known whether this seeming discrepancy can be explained by differences between PP5 and its

yeast homologue Ppt1, or by differences in GR regulation between yeast and mammalian cells in general.

The effects of the TPR proteins on SR observed here were significantly attenuated by saturating concentrations of hormone. This is consistent with an effect on hormone binding affinity. Different laboratories including ours provided evidence that later steps in steroid signal transduction are also affected by TPR cofactors, for example nuclear translocation [21,88,89] and dynamics of intranuclear mobility [90], which requires future experiments to clarify their relative contributions. Our results substantiate the concept that a delicate balance of TPR cofactors governs SR activity in a given cell or tissue, probably in a combinatorial fashion. The recent description of the N-terminal FKBP52 binding site on Hsp90 [85] opens the possibility for various combinations of two TPR proteins in the same SR heterocomplex. Alternatively, the dynamic assembly and disassembly of heterocomplexes may enable the sequential contribution of specific functions by the different TPR proteins.

## Materials and Methods

The MMTV-Luc reporter plasmid has been described previously [91]. ERE-Luc reporter plasmid and ER $\alpha$  and ER $\beta$  cDNA were a kind gift of Christian Behl (University Mainz). N-terminally HA-tagged ER $\alpha$  and ER $\beta$  were subcloned from ER cDNA into the pRK5-SV40 backbone. The plasmids expressing the N-terminally HA-tagged receptors GR, MR, PR and AR (pRK7 backbone) were kindly provided by Anke Hoffmann (MPI of Psychiatry, Munich) and the plasmid expressing Hsp90-FLAG by Len Neckers (NIH, Bethesda). The Gaussia-KDEL was constructed from a pCMV-GaussiaLuc1 plasmid (PJK) by linking the KDEL peptide sequence C-terminally via PCR, and subcloning into pRK5-SV40 backbone. TPR-proteins were all expressed as C-terminal FLAG-fusions in the pRK5-SV40 vector. The cDNA of CHIP was provided by Cam Patterson (University of North Carolina), of Cyp40 and TPR2 by Ulrich Hart (MPI of Biochemistry, Martinsried), of PP5 by Michael Chinkers (University of South Alabama). The plasmids expressing the FKBP51 and FKBP52 FLAG-fusions and the untagged FKBP52 in pRK5-SV40 were described previously [21]. The XAP2 plasmid is described in [37]. All plasmids were verified by sequencing. Primer sequences and cloning details are available upon request.

## Cell culture, transfection and reporter gene assay

Mouse embryonic fibroblasts (Marc Cox and David Smith, Mayo Clinic Scottsdale, Arizona, USA), human neuroblastoma SK-N-MC (ATTC HTB-10) and HEK-293 (ATTC CRL-1573) cells were cultured under conditions described previously [92,93]. For the MMTV-luc reporter gene assay, cells were seeded in 96 well plates (SK-N-MC 30,000 cells/well; MEF 10,000 cells/well) in medium containing 10% charcoal-stripped, steroid-free serum and cultured for 24 h before transfection using ExGen (Fermentas) as described by the manufacturer. Unless indicated otherwise, the amounts of transfected plasmids per well were 60 ng of steroid responsive luciferase reporter plasmid MMTV-Luc, 5–7.5 ng of Gaussia-KDEL expression vector as control plasmid, 25 ng of plasmids expressing HA-tagged steroid hormone receptors (mGR in case of MEF cells) and up to 300 ng of plasmids expressing TPR-domain containing cofactors (not exceeding 200 ng per single TPR-protein expression plasmid). If needed, empty expression vector was added to the reaction to equal the total amount of plasmid in all transfections. 24 h after transfection, cells were cultured in fresh medium supplemented with hormone as indicated or ethanol as control for 24 h. To measure reporter gene

activity cells were washed once with PBS and lysed in 50  $\mu$ l passive lysis buffer (0.2% Triton X-100, 100 mM  $K_2HPO_4/KH_2PO_4$  pH 7.8). Firefly and Gaussia luciferase activities were measured in the same aliquot using an automatic luminometer equipped with an injector device (Victor III, Wallac and Tristar, Berthold). Firefly activity was measured first by adding 50  $\mu$ l Firefly substrate solution (3 mM  $MgCl_2$ , 2.4 mM ATP, 120  $\mu$ M D-Luciferin) to 10  $\mu$ l lysate in black microtiter plates. By adding 50  $\mu$ l Gaussia substrate solution (1.1 M NaCl, 2.2 mM  $Na_2EDTA$ , 0.22 M  $K_2HPO_4/KH_2PO_4$ , pH 5.1, 0.44 mg/ml BSA, Coelenterazine 3  $\mu$ g/ml) the firefly reaction was quenched and Gaussia luminescence was measured after a 5 s delay. Firefly activity data represent the ratio of background corrected Firefly to Gaussia luminescence values. The fold stimulation reached at saturating concentrations of hormone was for GR about 1000, which is in the range of previous publications [65,91], MR 3.7, PR 970, AR 6, ER $\alpha$  4, and ER $\beta$  6.3. To compare the effects of co-expressed TPR proteins, the stimulation in the absence of the TPR protein was set to 100, and the stimulation in the presence of co-expressed TPR protein was referred to this value.

To check expression of receptors and TPR-proteins replicate lysates were pooled, briefly sonicated and cleared by centrifugation. Alternatively receptors and TPR-proteins were coexpressed in 6 well plates with the same receptor to TPR-protein ratios as for the 96 well plates. To this end, SK-N-MC cells were seeded in 6 well plates (500,000 cells/well) in medium containing 10% steroid-free serum and cultured for 24 before transfection of 0.25  $\mu$ g HA-tagged steroid hormone receptors and corresponding amounts of plasmids expressing TPR-proteins per well using ExGen (Fermentas) as described by the manufacturer. If needed, empty expression vector was added to the reaction to equal the total amount of plasmid in all transfections. Cells were cultured as for the reporter gene assay and lysed in buffer containing 20 mM Tris-HCl pH 6.8, 0.67% SDS, 3.3% Saccharose completed with Protease Inhibitor Cocktail (Sigma), briefly sonicated and cleared by centrifugation. Lysates were analyzed by SDS-PAGE followed by immunoblot.

### Statistical Analysis

To improve our understanding of the effects of various TPR proteins on steroid receptor mediated gene transcription, we performed one sample t-tests to evaluate the significance of difference of the hormone-stimulated activity of the receptor in the presence versus absence of coexpressed TPR protein. Significance values were corrected according to the Bonferroni procedure. The most pronounced differences with a significance level of  $p \leq 0.001$  are labelled in figures 3, 4 and 12 (\*).

### Immunoblot

Immunoblot detection of proteins was performed largely as described [94]. Briefly, proteins were transferred from SDS gels to a nitrocellulose membrane (Schleicher & Schuell, GmbH). Non-specific binding to membrane was blocked by 5% nonfat milk in Tris-buffered saline supplemented with 0.1% Tween-20, and then one of the following specific primary antibodies were added: Actin (I-19, Santa-Cruz), FLAG tag-HRP (M2, Sigma); hemagglutinin tag-HRP (Roche Applied Science); p23 (ABR), FKBP52 (Anti-FKBP59, Stressgen), CHIP (PC711, Calbiochem), Cyp40 (ABR, PA3-022), FKBP51 (F14, Santa Cruz), XAP2 (ARA9, NB100-127, Novus Biologicals), TPR2 (kind gift of Ulrich Hartl), PP5/PPT (BD Biosciences). Signals were visualized by appropriate secondary antibodies conjugated to horseradish peroxidase and the ECL system (Millipore, Billerica, USA) and documented on X-ray film.

### Immunoprecipitation

For immunoprecipitation of FLAG-tagged TPR proteins or HA-tagged steroid receptors, HEK-293 cells were transfected with 2–10  $\mu$ g of a plasmid expressing a TPR protein (amounts were adjusted to ensure comparable expression levels) and 5  $\mu$ g of a plasmid expressing a steroid receptor. For Hsp90 precipitations, 10  $\mu$ g of FLAG-tagged Hsp90 expression plasmid were transfected together with 10  $\mu$ g of FKBP52 plasmid. HEK 293 cells were chosen, because they efficiently expressed the proteins and showed the same results in reporter gene assays as SK-N-MC cells. Transfection was performed by electroporation of one confluent 10 cm (60 cm<sup>2</sup>) dish ( $\sim 5 \times 10^6$  cells) using a GenePulser (Bio-Rad, USA) at 350 V/700  $\mu$ F in 400  $\mu$ l of electroporation buffer (50 mM  $K_2HPO_4/KH_2PO_4$ , 20 mM KAc, pH 7.35, 25 mM  $MgSO_4$ ). Electroporated cells were replated in fresh medium containing 10% steroid-free serum containing medium and cultured for 3 days. Cells were harvested in cold PBS and lysed by resuspension in Lysis-Buffer A' (130 mM NaCl, 20 mM  $Na_2MoO_4$ , 1 mM EDTA, 20 mM Tris-HCl pH 7.5, 10% Glycerol, 0.5% Triton X-100, completed with Protease Inhibitor cocktail, Sigma) for FLAG-TPR protein and receptor HA-IP, or in Hsp90 Lysis Buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 20 mM  $Na_2MoO_4$ , 1 mM EDTA, 1 mM EGTA, 0.1% NP-40, 10% Glycerol, 0.5 mM DTT, completed with Protease Inhibitor cocktail, Sigma and Phosphatase Inhibitor cocktail, Roche) for the Hsp90 FLAG-IPs, followed by brief sonication (Branson Cell Disruptor B15, 3  $\times$  5 s, output 3) and incubation on ice for 1 h. The lysate was cleared by centrifugation (10 min, 25,000 rcf, 4°C) and the protein concentration was determined. 1–2 mg of lysate was incubated overnight at 4°C with the anti-FLAG M2 agarose affinity resin (Sigma) or with anti-HA agarose affinity resin (Sigma), respectively. FLAG-beads (30  $\mu$ l slurry) were treated as recommended by the manufacturer. The next day, the beads were washed 3 times with Lysis Buffer without detergent and samples were eluted with 70  $\mu$ l of 1  $\times$  FLAG-peptide solution (Sigma, 100–200  $\mu$ g/ml) or HA-peptide solution (Sigma, 100  $\mu$ g/ml), respectively, in 1  $\times$  Tris-buffered saline (150 mM NaCl, 10 mM Tris-HCl pH 7.0).

For analysis of the (co)precipitated proteins, 5–15  $\mu$ g of the cell lysates or 25  $\mu$ l of the immunoprecipitates were separated by SDS-PAGE under denaturing conditions. Coomassie staining was used for detection of immunoprecipitated TPR-proteins and coimmunoprecipitated Hsp90 and Hsp70 in the FLAG-IP. For all other detections immunoblots were used, i.e. the (co)precipitated steroid receptors in the FLAG- and HA-IP, co-precipitated FLAG tagged TPR-proteins in the HA-IP, and p23 in the FLAG-IP. To analyze relative binding, the signals were subjected to densitometry. The coomassie stained gels or films were scanned at 16 bit with a calibrated densitometer (GS800, Bio-Rad, USA) and analyzed with the Kodak 1D Image Analysis software.

To calculate the relative binding of co-precipitated proteins we proceeded as follows: For relative binding of the receptors to the precipitated TPR-proteins (FLAG-IP) the HA-immunoblot signals of the eluates were first normalized with the Coomassie density signals of the precipitated TPR proteins. To be able to compare results between different experiments, we calculated these data to represent relative receptor binding among the TPR proteins. To this end, the normalized receptor (HA-IB) signal for each TPR protein was divided by the mean of the normalized receptor signals of all TPR proteins in each experiment. These ratios could then be averaged throughout the different experiments.

Conversely, to calculate the relative binding of the associated TPR proteins to the precipitated receptors (HA-IP), the FLAG-immunoblot signals of the HA-IP eluates were normalized first

with the HA-immunoblot signals of the HA-IP eluates (to correct for variations in precipitation efficiencies of the receptors), and second to the FLAG-immunoblot signals of the lysate (to correct for differences in TPR protein expression). To calculate the mean of different experiments, like for the FLAG-IP, the normalized signals of each TPR protein were represented in reference to the relative binding of the other TPR proteins. Because of variabilities of the HA-IPs, the relative binding of each TPR protein were not normalized to the mean of all TPR proteins, but for each receptor to the mean of a subset of TPR proteins. The subset of TPR proteins used to calculate the mean binding in different experiments are displayed in the figure legends of each receptor.

To analyze the relative binding of Hsp90, the Hsp90 (FLAG-IP)-Coomassie signals were normalized to the Coomassie signals of the precipitated TPR proteins and this relative binding was used to calculate the mean binding in different experiments.

To analyze relative Hsp70 binding to TPR proteins (FLAG-IP), first the Hsp70 signal of the control reaction (= background Hsp70 binding) was subtracted from the Hsp70 coomassie signals of each TPR protein, and these values were then normalized to the

coomassie signals of the precipitated TPR proteins. Slightly negative values were considered as no binding and set to zero. This relative binding was used to calculate the mean binding in different experiments. To analyze significant binding of Hsp70 to FKBP51, a two tailed heteroscedastic students t-test were applied.

## Acknowledgments

The authors thank Kathrin Hafner for outstanding technical assistance, Anja Kretzschmar for excellent support with MR reporter gene experiments, Cam Patterson, Anke Hoffmann, Christian Behl, Osborne Almeida, Len Neckers and Ulrich Hartl for kindly providing cDNAs and plasmids, David Smith and Marc Cox for kindly providing wt and FKBP52 KO MEF cells, Jürgen Zschocke for critical reading of the manuscript, and Florian Holsboer for continuous support.

## Author Contributions

Conceived and designed the experiments: JPS GMW TR. Performed the experiments: JPS GMW ILR NCG BB. Analyzed the data: JPS GMW ILR AY TR. Contributed reagents/materials/analysis tools: RTK. Wrote the paper: JPS TR.

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